The AMPK-PRC2 Signalling Axis and Energy Stress Gene Expression in Non-Small Cell Lung Cancer

Master's Thesis

Minea Saikkala

International Master's Degree Program in Translational Medicine

Faculty of Medicine

University of Helsinki

2021

Table of contents

Abbreviations

ACC	Acetyl-CoA carboxylase
ADC	Adenocarcinoma
AMPK	AMP-dependent kinase
ATF3	Activating transcription factor 3
BSA	Bovine serum albumin
CAMK2A	Calcium/calmodulin dependent protein kinase II alpha
cDNA	Complementary DNA
DMED	Dulbecco's Minimum Essential Medium
DNMT	DNA methyltransferase
dNTP	deoxyribose nucleoside triphosphate
EED	Embryonic ectoderm development
EZH2	Enhancer of zeste homologue 2
FBS	Foetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
HES1	Hairy and enhancer of split-1
H3K27ac	Histone 3 lysine 27 acetylation
H3K27me3	Histone 3 lysine 27 trimethylation
JMJD3	Jumonji domain-containing protein D3
KDM6A/B	Lysine demethylase 6A/B
KLF4	Krüppel-like factor 4
KRAS	Kirsten rat sarcoma viral oncogene homolog
LKB1	Liver kinase B1
lncRNA	Long non-coding RNA
MEF	Mouse embryonic fibroblast
NSCLC	Non-small cell lung cancer

OAZ1	Ornithine decarboxylase antizyme 1
p-ACC	Phospho-ACC
p-AMPK	Phospho-AMPK
PBS	Phosphate-buffered saline
PcG	Polycomb group proteins
PIM1	Proto-oncogene serine/threonine protein kinase Pim-1
PJS	Peutz-Jeghers syndrome
PRC1/2	Polycomb repressive complex 1/2
PRKAA/B/G	Protein kinase AMP-activated catalytic subunit alpha/beta/gamma
RbAp46/48	Retinoblastoma protein associated protein 46/48
RT-qPCR	Real time quantitative polymerase chain reaction
SCC	Squamous cell carcinoma
SCLC	Small cell lung cancer
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SOX2	Sex determining region Y box 2
SUZ12	Suppressor of zeste 12 homolog
TBS	Tris-buffered saline
Thr	Threonine
TP53	Tumour protein P53
UTX	Ubiquitously transcribed tetratricopeptide repeat X chromosome
wt	Wild type
2-DG	2-deoxyglucose

Abstract

Lung cancer is one of the most common and deadliest cancers worldwide, but the mechanisms behind different types of lung cancer are still poorly understood. Non-small cell lung cancer makes up 80% of lung cancers, and some epigenetic mechanisms have been proposed for it. Epigenetic modifications are a way of influencing the expression of genes by inhibition or activation. PRC2 is an epigenetic modulator that catalyses the formation of methyl groups on histone 3 lysine 27, which is an epigenetic mark with repressive nature. PRC2 has been proposed to be downstream of AMPK, an energy sensor of the cell, which is phosphorylated by LKB1 under energy stress conditions. Inactivating mutations in LKB1 are known to cause and worsen non-small cell lung cancer, and the overexpression of EZH2, the catalytic subunit of PRC2, has similar effects. Therefore, establishing a novel downstream mechanism linking LKB1, AMPK, and PRC2 together could explain one mechanism for NSCLC tumorigenesis. Changes in metabolism are a feature of cancer cells, and this pathway could also link energy stress and cancer together.

Mouse embryonic fibroblast and H358 cell lines overexpressing wild type EZH2, mutant EZH2 and GFP were generated and treated with the glycolysis inhibitor 2-deoxyglucose to study the effects of energy stress. Levels of histone methylation and phosphorylation statuses of AMPK and its downstream target ACC were assessed with Western blotting, and expression levels of potential PRC2 target genes with RT-qPCR. The study setting proved to be functional for the response of AMPK to energy stress conditions, as both AMPK and ACC were phosphorylated in the presence of 2-DG. In mouse embryonic fibroblasts, PIM1 showed different gene expression with wild type and mutant EZH2, suggesting that its activation would be regulated through the phosphorylation of the T311 site of EZH2 during energy stress.

The results from histone methylation statuses did not follow the hypothesis, possibly because of the lack of specificity of detecting global H3K27me3. Other target genes besides PIM1 in MEFs did not show significant changes in expression level. Considering that the incorporation of the mutant EZH2 into PRC2 complexes was not validated, additional research would be needed to confirm or deny the explained mechanism between PRC2 and AMPK.

Introduction

Non-small cell lung cancer

Cancer causes the second most deaths worldwide, and lung cancers are among the most common cancers both in men and women. One-quarter of all cancer deaths are due to lung cancer, and at 19%, lung cancer still has the lowest 5-year survival rate across all cancers (Siegel et al. 2019). Therefore, there is a need to find possible drug targets and increase the understanding of the mechanisms behind lung cancer.

This thesis focuses on non-small cell lung cancer (NSCLC), which accounts for 80% of lung cancers. NSCLC consists of adenocarcinoma, squamous cell carcinoma and large cell carcinoma. Known causes that contribute to the rise of lung cancer include tobacco smoke, ionising radiation, and viral infections, but the mechanisms are not yet well established (Esposito et al. 2010). Many mechanisms behind cancer and NSCLC can be attributed to epigenetics, including the mechanism that will be studied in this thesis.

Epigenetics

Epigenetics is broad field of study that was initially used to explain the hereditary phenomena that could not be explained by basic genetics. It has been studied intensively in the last decades, as it helps to explain the gap between genotype and phenotype. With epigenetic modifications, it is possible to change the way genes are expressed without changing the nucleotide sequence of the genome. The epigenome is semi-dynamic, meaning that environmental factors can change it, but some of those changes are heritable. Epigenetic modifications include DNA methylation, histone variants, noncoding RNA, and chromatin modifications (Goldberg et al. 2007), which will be discussed below.

DNA methylation

DNA methylation is found on cytosine residues of CpG islands and its function is related to transcriptional repression. CpG islands are regions of the genome that have exceptionally high amounts of cytosine and guanine nucleotides (Goldberg et al. 2007). The methyl group is covalently attached to the 5’ position of the cytosine by methyl transferases such as DNMT3A, DNMT3B and DNMT1, making it a 5-methylcytosine. DNA methylation silences genes, and in fact most

transcriptional silencing of human genes occurs via DNA methylation. DNMT3A/B carry out de novo methylation, and DNMT1 conserves methylation patterns across cell divisions. This enables for the methylation pattern of the genome to be both dynamic and heritable, as environmental cues drive new methylation and it can be passed on in cell divisions (Mazzio et al. 2012).

Histone variants

Genomic DNA resides inside the mammalian cell nucleus, packaged into units called nucleosomes. A core nucleosome contains a histone octamer and DNA wrapped around it. The histone octamer is composed of two of each histone proteins H2A, H2B, H3 and H4. Linker histone H1 stabilises core nucleosomes into higher order structures, which makes the DNA 30 to 40 times more compact (Luger et al. 1997). There are several different histone variants, and their variation has an impact on epigenetics. Although nucleosomes are tightly packed, their function can be both activating and deactivating to gene expression, mostly through post-translational modifications on histone amino acid residues (Kimura 2013).

Non-coding RNA

Although non-coding RNAs (ncRNAs) were thought to be non-functional for a long time, numerous studies have proven them to be important regulators of gene transcription and translation. Long non-coding RNAs (lncRNAs) have been found to participate in the silencing of genes and are thus considered to constitute an important epigenetic mechanism. For instance, lncRNAs regulate transcription through epigenetic modifications by interfering with chromatin remodelling complexes such as the Polycomb repressive complexes PRC1 and PRC2. An example of an important lncRNA is the silencing of the inactive X chromosome, which is mediated through the expression of X-inactive specific transcript (XIST) (Saxena et al. 2011).

Chromatin modifications

Histone modifications are covalent additions to the N-terminal tail of the histone. These changes alter the charge of the tail residues and change the conformation of the histone and its affinity to DNA and other chromatin-binding molecules. The number of different histone mark combinations is extremely high, as modifiable amino acid residues include lysine, serine, tyrosine and arginine, and possible modifications include methylation, acetylation, phosphorylation, ubiquitination, biotinylation, sumoylation and proline isomerization (Mazzio et al. 2012). Abbreviation are commonly used for histone modifications, following the pattern of first the histone, then the amino

acid residue, and last the modification. For example, H3K27me3 stands for histone 3 lysine 27 trimethylation.

Although not all histone marks can be linked to a specific function, methylation and acetylation patterns of lysine residues of histone H3 can usually give reliable information about the transcriptional activity of the gene in question. The methylation status of lysine residues can be mono-, di- or trimethylation, and it cannot coexist with acetylation on the same lysine. The effect of methylation on H3 is variable, the effect of the methylation depending on the lysine residue and the methylation level. Specific histone modifications can still generally be linked to either gene activation or repression (Kimura 2013).

Trimethylation of histone 3 lysine 27 is a mark associated with gene repression and it is often found in silenced chromatin such as the inactivated X chromosome (Nozawa et al. 2013). H3K27me3 is catalysed by the Polycomb repressive complex 2 (PRC2), a protein complex that contains the methyltransferase EZH2 (Margueron et al. 2011). Demethylation of H3K27me3 is done by KDM6A/UTX and KDM6B/JMJD3 (Sen et al. 2008). Abnormal regulation of both the methyltransferase and demethylases are associated with cancer (Albert et al. 2010; Pedersen et al 2010).

Polycomb group proteins

Polycomb repressive complex 1 and 2 (PRC1/2) are complexes formed by Polycomb group proteins (PcG). PcG proteins are associated to silent chromatin states and their counterpart Trithorax group proteins (TrxG) are associated to active chromatin states (Schuettengruber et al. 2007). PcGs are important gene regulators at all stages of cell development and are major regulators of cell identity. Activity of some PcG members can extend the lifespan of epithelial cells by inducing telomerase activity, while some control cellular proliferation and tumorigenesis. This highlights the importance of epigenetic control during different stages of development (Ringrose et al. 2004).

In mammals, PRC2 is composed of its core components EZH2, EED, SUZ12 and RbAp46/48 (Schuettengruber et al. 2007). PRC2 is a methyltransferase that catalyses the mono-, di-, and trimethylation of lysine 27 of histone H3. It is a basic epigenetic function that maintains gene expression patterns in normal cells, and is involved in differentiation, proliferation and maintaining cell identity, among others (Laugesen et al. 2019; Margueron et al. 2011). The trimethyl mark

H3K27me3 made by PRC2 has been studied the most, and it is widely accepted as a mark of gene repression (Laugesen et al. 2019). PRC2 catalyses the formation of the methylation marks through its catalytic subunit, the methyltransferase Enhancer of zeste homologue 2 (EZH2). EZH2 is only present in cells that are actively dividing, as opposed to EZH1 which is found in both dividing and differentiated cells. PRC2 complexes that have EZH2 have higher methyltransferase activity than those with EZH1 (Margueron et al. 2011).

The involvement of PRC2 in cancer is usually due to the dysregulated expression of its subunits, especially EZH2 overexpression. Other subunits have also been found to be overexpressed in cancers, including NSCLC. In colorectal cancer patients, expression of EZH2, EED and SUZ12 mRNA was significantly increased when compared to non-cancerous mucosa tissue. Higher levels of these PRC2 subunits also correlated with worse prognosis and aggressive clinical behaviour (Liu Y.L. et al. 2015). SUZ12 alone has also been suggested to be an oncogene in multiple cancers, including non-small cell lung cancer. An increase in SUZ12 expression was found in NSCLC, and it was suggested to promote growth and metastasis of cancer cells (Liu et al. 2014).

EZH2

EZH2 is expressed in rapidly proliferating and developing tissue during embryogenesis. It is mainly found in undifferentiated cells (Ezhkova et al. 2009). An overexpression of EZH2 in differentiated cells will thus revert the cell back to rapid proliferation and other stem-like characteristics, which is favourable for the initiation of cancer. EZH2 has indeed been found to be overexpressed or mutated in a large variety of cancers, such as prostate, breast, bladder, endometrial cancer and melanoma. Deregulation of EZH2 also usually correlates with poor prognosis, aggressiveness, or advanced stages of the cancer (Dimou et al. 2017).

In breast cancer, EZH2 levels correlate with the aggressiveness of the cancer, and EZH2 overexpressing tumours are also more likely to metastasize (Kleer et al. 2003). In prostate cancer, the overexpression of both EZH2 mRNA and protein correlate with a poor prognosis, and EZH2 is the most upregulated gene in metastatic prostate cancer (Varambally et al. 2002). In addition to sporadic mutations in EZH2, the Polycomb machinery can also be directly engaged by an environmental carcinogen. For example, tobacco smoke activates the cancer stem cell maintenance machinery through the recruitment of EZH2 and SUZ12 and the hypermethylation of tumour

suppressor promoters (Hussain et al. 2009). Oestrogen is also an environmental signal that can repress EZH2 and reduce the H3K27me3 mark in chromatin, causing uterine leiomyomas (Greathouse et al. 2012).

Abnormalities in EZH2 expression have been detected in different cancers. Various studies have also found an overexpression of EZH2 in non-small cell lung cancers. When testing for EZH2 in NSCLC tissue and normal tissue, EZH2 was found to be overexpressed in NSCLC tissue. This overexpression also correlated with poor prognosis. In addition, EZH2 was found to enhance cell growth and increase the progression of the cell cycle (Takawa et al. 2011). In another set of NSCLC tissue samples, squamous cell carcinoma showed higher expression of EZH2 than adenocarcinoma, and the expression of EZH2 in adenocarcinomas correlated with young age and smoking. NSCLC brain metastases were also found to have higher EZH2 than primary tumours. High EZH2 expression was found to predict aggressive tumour behaviour and rapid metastasis development (Behrens et al. 2013).

The effects of EZH2 overexpression have also been studied in a mouse model. The overexpression did not affect the mice during development, but the mice developed multiple different tumours in adulthood, including lymphoma, liver sarcoma and lung adenocarcinoma. An overall overexpression of EZH2 caused half of the mice to develop lung adenocarcinoma, and a targeted overexpression of EZH2 in the lung caused 42% of the mice to develop lung adenocarcinoma. EZH2 overexpressing tumours were also found to have decreased H3K27ac and increased H3K27me3, and a suppression of a set of EZH2 target genes was detected. This study also proposed an EZH2 inhibitor, which had anti-tumour activity (Zhang et al. 2016). Taking these results together, EZH2 could be used both as a diagnostic tool and a therapeutic target in NSCLC.

The activity of EZH2 is mostly PRC2-dependent (Wassef et al. 2019). However, EZH2 overexpression cannot be treated as an increased activity of PRC2. There is some evidence of an independent role for EZH2. Solo EZH2 can act as a coactivator for other transcription factors and has been shown to have oncogenic function in prostate cancer (Xu et al. 2021). PRC2-independent EZH2 also acts in breast cancer by promoting cell proliferation (Shi et al. 2007) and activating or repressing transcription (Lee et al. 2011). However, these functions are not related to the H3K27me3 mark, but rather to H3K4me2 and H3K4me3 (Xu et al. 2012), implying that the target genes of solo EZH2 and PRC2-dependent EZH2 are most likely different.

The possible link between PRC2 and energy stress

It has been proposed that an upstream regulator of PRC2 would be AMPK, which is a kinase that senses the energy status of the cell and responds to energy stress by being phosphorylated by LKB1. According to Wan et al. active AMPK would be able to phosphorylate the catalytic subunit EZH2. This phosphorylation would disrupt the connection between EZH2 and SUZ12, rendering the PRC2 complex unable to catalyse the trimethylation of lysine 27 of histone 3. Genes normally repressed by this epigenetic mark would then be activated (Wan et al. 2018). If PRC2 is downstream of LKB1 and AMPK, the activity of PRC2 would be changed according to the energy status of the cell, and the target genes of PRC2 would likely relate to metabolic pathways.

AMPK

AMP-dependent kinase (AMPK) is one of the main energy level sensors in the cell. It is usually found as a heterotrimeric protein complex, and its subunits are well conserved throughout all eukaryotes. AMPK is composed of the catalytic α-subunit and the regulatory β- and γ-subunits, all of which have paralogous genes expressing distinct isoforms of each of the subunits (AMPK-α1, -α2; -β1, -β2; -γ1, -γ2, -γ3, encoded by *PRKAA1*, *PRKAA2*; *PRKAB1*, *PRKAB2*; *PRKAG1*, *PRKAG2*, *PRKAG3*, respectively) (Hardie 2015). The different isoforms may change the function of the complex or have different subcellular locations. The isoforms of catalytic subunit are very similar yet have different substrate specificity and have a different phenotype as knockouts (Ross et al. 2016).

AMPK functions by sensing changes in cellular AMP/ATP ratio, as the ratio rises during energy stress such as energy deprivation or exercise. When AMPK is activated by energy stress, it catalyses changes in the energy metabolism of the cell. AMPK senses the energy status of the cell with its γ-subunits. They contain three binding sites for AMP, with ATP and ADP competitively binding to at least two of these sites. When the cell is under energy stress, less ATP and ADP is available, and the binding sites are occupied mainly by AMP (Hardie 2015). The binding of AMP induces a conformation change that makes AMPK a better substrate for liver kinase B1 (LKB1), the AMPK activating kinase, which then phosphorylates the Thr172 residue in the activation loop of the α subunit of AMPK (Young 2009). The binding of AMP also inhibits the dephosphorylation of AMPK Thr172 by protein phosphatases and activates AMPK by allosteric activation (Gowans et al. 2013).

AMPK changes the metabolism of the cell when activated, and the activation requires the kinase activity of LKB1, which is upstream of AMPK. The specialty of this activation process is that LKB1 is

not affected by the energy status of the cell. This is important because it is an upstream kinase to multiple kinases with different functions. It is constitutively active, and the changing energy level has an impact on AMPK, not LKB1. This way AMPK is only phosphorylated when the conditions are right, even though LKB1 is always active (Lizcano et al. 2004).

When activated, AMPK promotes catabolic pathways that produce ATP and inhibits anabolic pathways that require ATP. Catabolic pathways are, for example, glucose uptake and glycolysis, fatty acid uptake and synthesis and mitochondrial biogenesis. Anabolic pathways that are inhibited include fatty acid oxidation, rRNA synthesis, cellular growth, and division, which take up energy (Hardie et al. 2012). The role of AMPK is not limited to cellular level, as it also acts on systemic level. For example, AMPK increases food intake when expressed in the hypothalamus, increases fatty acid oxidation in skeletal muscle (Minokoshi et al. 2002) and suppresses gluconeogenesis in the liver (Yamauchi et al. 2002).

Mechanistically, AMPK achieves these wide regulatory functions by phosphorylating a large variety of target proteins. Upon activation, AMPK phosphorylates TBC domain family member 1 (TBC1D1) and thioredoxin-interacting protein (TXNIP) to promote glucose uptake into the cell. AMPK also phosphorylates 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) to regulate glycolysis and phosphorylates cyclic-AMP-regulated transcriptional co-activator 2 (CRTC2) and class II histone deacetylases (HDACs) to inhibit the transcription of genes involved in gluconeogenesis. In addition, AMPK inhibits protein synthesis by inhibiting the mechanistic target of rapamycin complex 1 (mTORC1), which functions in the activation of biosynthetic pathways such as protein translation and cell growth. Moreover, AMPK regulates mitochondrial processes such as mitophagy, mitochondrial fission, and mitochondrial biogenesis to enhance metabolic efficiency (Garcia et al. 2017). AMPK is a kinase with many phosphorylation targets and important impacts on the energy metabolism of cells and tissues. Metabolic enzymes and metabolites can also regulate gene expression both directly and indirectly by activating or repressing other regulators. Therefore, AMPK can regulate gene expression through metabolic changes. Taking glucose metabolism as an example, it affects the expression of glycolytic and lipogenic enzymes, insulin, and glucose transporters in the liver and muscle (Sukumaran et al. 2020).

AMPK in cancer

An abnormal metabolism is a hallmark of cancer. Cancer cells use aerobic glycolysis instead of oxidative phosphorylation. This is called the Warburg effect (Hanahan et al. 2000). In cancer cells, nutrient deprivation drives the metabolism of the cell towards the Warburg effect via a pathway that involves AMPK. This rescues the cells from apoptosis during nutrient deprivation (Wu et al. 2013). Loss of AMPK has also been found to drive this switch from oxidative phosphorylation to aerobic glycolysis. The ability of AMPK to change the metabolism of the cell could be the mechanism through which AMPK carries out tumour suppressor functions. When AMPK is inactive, the metabolism of the cell shifts towards aerobic glycolysis, biomass accumulation and an increased amount of glucose carbons used in lipid synthesis. The pathways that are active when there is no or little AMPK in the cell enhance cell growth and proliferation, thus giving cancer cells a growth advantage (Faubert et al. 2013). In addition, AMPK has tumour suppressors both upstream and downstream in its signalling pathway. Well-known tumour suppressors include LKB1 upstream (Shaw et al. 2004) and TSC2 downstream of AMPK (Inoki et al. 2002).

Surprisingly many cancer-driving mutations also have an impact on cell metabolism, suggesting that oncogenes and tumour suppressors are involved in metabolic pathways (Levine et al. 2010). The same mutations that give rise to cancer often also control the metabolism of the cancer cells, which is beneficial to the proliferation and growth of transformed cells (Shaw 2006).

AMPK has indeed been found to be dysfunctional in breast cancer, where the signalling of AMPK phosphorylation was reduced (Hadad et al. 2009). The reduced expression of the gene that encodes AMPKα2 subunit (PRKAA2) was suggested to be a key modulator in the tumorigenesis of gastric cancer, as it was found to be differentially expressed, often mutated, and a target gene of key miRNAs (Kim et al. 2012).

AMPK activity may also result in a pro-cancer situation. By changing the metabolism of the cell in nutrient-sparse situations, it helps the cells to adapt to such conditions and protects the cell from cellular stress due to energy deprivation. When AMPK functions normally and the cell encounters energy stress, AMPK blocks cellular growth and helps to activate alternative pathways for energy production (Shaw et al. 2004). Therefore, loss of AMPK may result in apoptosis under energy stress or hypoxic conditions (Faubert et al. 2013). Consequently, inhibition of AMPK combined with metabolic inhibitors could result in apoptosis, which could be explored as a therapeutic strategy for cancer. The same effect has been observed in LKB1 deficient tumour cells, which go into apoptosis

under energy stress conditions. The LKB1/AMPK signalling protects the cell against apoptosis when AMP levels are low (Shaw et al. 2004). Therefore, cancer treatment could benefit from the cancer cell's dependence on AMPK in relation to energy stress.

LKB1

Liver kinase B1 (LKB1 or STK11) is a tumour suppressor that was initially discovered alongside the Peutz-Jeghers syndrome, caused by LKB1 mutations. These patients develop benign hamartomatous polyps in the gastrointestinal tract and are also at a very high risk of developing malignant tumours in multiple tissues. Sporadic mutations in LKB1 are behind 10-20% of PJS cases (Alessi et al. 2006) but are also found in one third of lung adenocarcinoma patients (Sanchez-Cespedes et al. 2002).

In lung cancer, LKB1 is the third most commonly mutated gene after TP53 and KRAS (Blanco et al. 2009). In non-small cell lung cancer, 80% of cell lines have loss of heterozygosity at the LKB1 locus in chromosome 19p (Virmani et al. 1998). Deletions in LKB1 have also been suggested to drive lung adenocarcinomas subtype switching to more aggressive squamous cell carcinomas (Zhang et al. 2017). Alterations in LKB1 are more common in NSCLCs than SCLCs (Matsumoto et al. 2007) and are especially common in lung adenocarcinomas (Carretero et al. 2004). Some mutations in LKB1 hamper its ability to phosphorylate substrates such as AMPK (Hawley et al. 2003), resulting in reduced ability of responding to energy stress.

The possible link between AMPK and PRC2 would establish a pathway for LKB1 causing non-small cell lung cancer. An inactive LKB1 leads to AMPK inactivation. Without the phosphorylation of EZH2 by AMPK, the PRC2 complex could stay active in all energetic situations, keeping target genes repressed in situations of energy stress. This could be the mechanism by which inactivating LKB1 mutations lead to worse cases of non-small cell lung cancer.

Target genes

The target genes for this study were chosen from an RNA sequencing study performed in the laboratory of Tomi Mäkelä, where they were found to be induced by 2-deoxyglucose (2-DG) treatment in mouse embryonic fibroblasts and to be AMPK dependent (unpublished data). 2-DG is a glucose analogue and an inhibitor of glycolysis, taken up by the cell and phosphorylated by hexokinase in a manner similar to normal glucose, but the product 2-deoxyglucose-6-phosphate

cannot proceed into glycolysis. This causes a decrease in energy produced from glucose and the activation of AMPK, and results in energy stress. The gene expression data set resulting from the RNA sequencing study was discovered in a gene set enrichment analysis (GSEA) to resemble previously published data sets investigating PRC2 function and H3K27me3 marked chromatin. In addition, there is evidence in literature that they could be PRC2 targets.

ATF3

Activating transcription factor 3 (ATF3) is an adaptive-response gene and part of the ATF/cyclic AMP response element-binding transcription factor family. Its function is to adapt the cell to extra- and intracellular changes by activating or repressing gene expression. Due to its role in the regulation of cell cycle and apoptosis, ATF3 has been studied in relation to oncogenesis. Depending on the context, it seems to be able to act as both an oncogene and a tumour suppressor (Thompson et al. 2009). There is evidence of ATF3 overexpression in human cancers, such as breast cancer (Yin et al. 2008) and malignant prostate cancer with poor prognosis (Pelzer et al. 2006), but there is also evidence of ATF3 overexpression increasing apoptosis and reducing metastatic potential of human cancer cells in prostate cancer (Huang et al. 2008) and ovarian cancer (Syed et al. 2005).

The link between ATF3 and lung cancer remains unclear. In one study, NSCLC cells were compared with normal bronchial epithelial cells, and an overexpression of ATF3 was found in the NSCLC cells (Song et al. 2012). However, in another study a downregulation of ATF3 was reported in lung cancer tissue specimens, and it correlated with metastasis (Jan et al. 2012). Evidence for ATF3 being a target of PRC2 also has not been studied much, but one study in T helper 15 cells found ATF3 to be a direct target of PRC2 (Escobar et al. 2014).

KLF4

Krüppel-like factor 4 (KLF4) is a transcription factor that regulates processes such as cell growth, proliferation, and differentiation (Ghaleb and Yang 2017). Therefore, it also plays a role in cancer, where its activity is regulated by hypermethylation of the KLF4 promoter (Cho et al. 2007) and histone methylation (Lindeman et al. 2010). In normal conditions, KLF4 suppresses apoptosis, but can switch its role to anti-apoptotic under certain conditions. KLF4 also has a dual role in cancer, as it has been found to function both as a tumour suppressor and an oncogene, depending on the cell and cancer types. This means that both less of KLF4 expression and overexpression of KLF4 have been found in cancers (Ghaleb and Yang 2017). This duality can be seen even within lung cancers, as KLF4 expression was decreased in non-small cell lung cancer but overexpressed in small-cell lung

cancer (Fadous-Khalifé et al. 2016). The lower expression levels of KLF4 in lung cancer were found to be both at protein and RNA level, and KLF4 was found to be methylated in more tumour tissues than normal tissues (Hofstetter et al. 2009). As hypermethylation is connected to transcriptional repression, KLF4 could be regulated in NSCLC through its methylation status.

The role of KLF4 as an oncogene or as a tumour suppressor might be related to its subcellular localisation. Higher levels of KLF4 in the nucleus of a cell were linked to a worse prognosis for NSCLC patients, whereas higher levels in the cytoplasm correlated with better prognosis. These two were independent of each other (Liu et al. 2018).

There is some evidence that EZH2 could be an upstream regulator of KLF4. In one study, the silencing of EZH2 was found to upregulate KLF4. EZH2 catalysing the formation of a silencing mark on the KLF4 promoter produced less KLF4. This was linked to poor prognosis in ovarian cancer (Zhang et al. 2019). Considering these observations, downregulation of KLF4 in relation to non-small cell lung cancer could be worth researching.

PIM1

PIM1 is a serine/threonine kinase that participates in the regulation of cell survival, proliferation, and differentiation. It is a constitutively active kinase that can be phosphorylated for stability (Shah et al. 2008). PIM1 is a proto-oncogene that has a role in the tumorigenesis of different cancers, as it is overexpressed in prostate carcinomas (Valdman et al. 2004) and the chromosome region where Pim1 resides is often mutated in haematopoietic malignancies (Betts et al. 2008) and in non-small cell lung cancer (Kim et al. 2005). PIM1 is expressed in normal human cells, but its overexpression correlates with tumour aggressiveness and poor prognosis. A mutation in the PIM1 gene has been observed in NSCLCs, and it correlated with poor survival (Shah et al. 2008). Other studies also link higher expression of PIM1 to NSCLC by proposing that PIM1 could be involved in the tumorigenesis or the progression of non-small cell lung cancer (Jin et al. 2012) and that it could be a mediator of radioresistance in NSCLC (Kim et al. 2013).

One proposition on how PIM1 could participate in tumorigenesis is metabolism. PIM1 was found to promote the Warburg effect in cancer cells, and it was upregulated in response to AMPK signalling. Phosphorylated AMPK and PIM1 expression increased correspondingly under glucose deprivation, and PIM1 expression was induced by AMPK activator A769662 treatment (Zhang et al. 2018). LKB1-AMPK signalling could thus be a factor in the development of non-small cell lung cancer partly by

activating PIM1. There is almost no evidence that PIM1 would be a target of PRC2. One study found PRC2 binding to PIM1 RNA in mouse embryonic stem cells (Beltran et al. 2019). This is, however, not enough evidence that PRC2 would regulate the expression of PIM1.

HES1

Hairy and enhancer of split-1 (HES1) is a transcription factor that participates in physiological processes such as cell cycle and cellular differentiation, apoptosis, and self-renewal. Due to these properties, HES1 is also involved in cancer progression. It has been found to function in metastasis and tumour multidrug resistance (Liu Z.H. et al. 2015).

HES1 has also been found to play a role in non-small cell lung cancer. HES1 overexpression was statistically associated with lower overall survival rate in both lung adenocarcinoma and lung squamous cell carcinoma. This association is likely due to Notch signalling, which is often higher in NSCLC and predicts poor prognosis. HES1 is a target of the Notch pathway, explaining why both are elevated in NSCLC (Yuan et al. 2015).

Due to HES1 being a well-known direct transcriptional target of NOTCH1 (Palomero et al. 2006), a link between PRC2 and NOTCH1 most likely also links PRC2 and HES1 together. Indeed, in a study on in T cell acute lymphoblastic leukaemia, HES1 was upregulated in experiments where PRC2 subunits EZH2 and SUZ12 were silenced. In addition, H3K27me3 levels were found to inversely correlate with HES1 expression (Ntziachristos et al. 2012).

SOX2

Sex determining region Y box 2 (SOX2) is a transcription factor well known for its stem cell properties, such as self-renewal and pluripotency. It also plays important roles in cell cycle control and DNA damage response. SOX2 is therefore also able to maintain stem cell-like properties in cancer cells. This has been studied in non-small cell lung cancer, where the overexpression of SOX2 stimulates cell migration and anchorage-independent growth. In addition, when SOX2 was silenced in NSCLC cell lines, apoptosis was induced (Chen et al. 2013). A link of SOX2 to the progression of tumours in NSCLC has also been reported (Velcheti et al. 2013; Sholl et al. 2010). However, there have been conflicting findings about the overexpression of SOX2. Some studies show that upregulated SOX2 gene amplification correlated with poor prognosis (Sholl et al. 2010), whereas according to other studies, high levels of SOX2 would correlate with better prognosis (Velcheti et al. 2013). In lung cancers, SOX2 has been found to be amplified in squamous cell carcinomas and linked

to poor prognosis in adenocarcinomas (Bass et al. 2009). There are also differences in the upregulation status of SOX2 in SCC and ADC, with it being more expressed in SCC (Chen et al. 2013).

Some studies indicate that SOX2 and PRC2 could be related. The regulatory regions of SOX2 were found to be occupied by H3K27me3 and EZH2, which happened via the Calcium/calmodulin dependent protein kinase II alpha (CAMK2A). The suppression of CAMK2A increased EZH2 binding to the SOX2 regulatory regions and increased the H3K27me3 mark, leading to the reduced expression of SOX2 (Wang et al. 2020). In addition, other studies have suggested that SOX2 would collaborate with PRC2, co-occupying promoters of downstream developmental genes (Guo et al. 2018). SOX2 has also been connected to LKB1 and AMPK. Loss of LKB1 and overexpression of SOX2 are suggested to promote lung squamous cell carcinoma. Consistent with the fact that AMPK is a substrate of LKB1, an absence of phosphorylated AMPK was found in SOX2-LKB1 tumours (Mukhopadhyay et al. 2014).

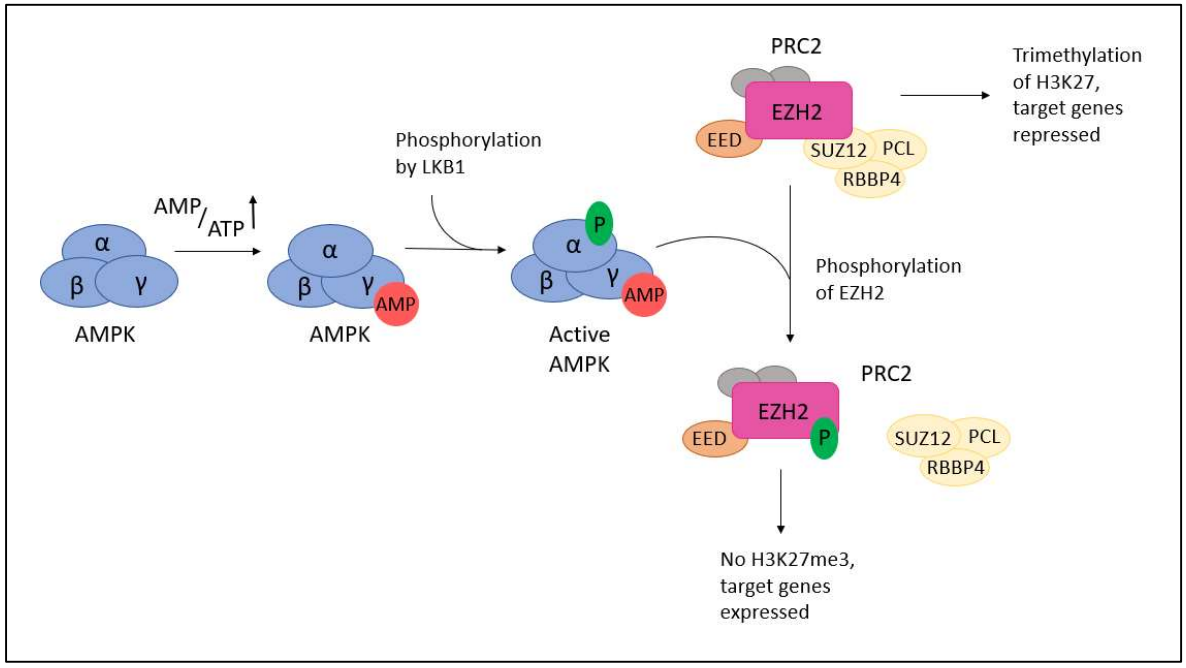
Aims of the study

The aim of this project is to investigate the relationship between non-small cell lung cancer and the AMPK/PRC2 signalling axis during energy stress. This study aims to validate the mechanism of AMPK dependent transcriptional regulation by PRC2 and to identify genes regulated by the AMPK-PRC2 signalling pathway in cellular energy stress.

The study will strengthen the knowledge on mechanisms by which AMPK regulates metabolism during energy stress. EZH2 has been identified to be a potential target for anticancer therapy (Deb et al. 2014), but there is still very limited information of the cellular mechanisms of AMPK and PRC2. LKB1 mutations are common in NSCLC (Virmani et al. 1998), and finding a downstream mechanism linking AMPK and PRC2 together could help understand how LKB1 inactivation drives non-small cell lung cancer. Therefore, understanding how energy stress affects the activation of genes has clinical significance.

Metabolism has an important effect on cancer, and their relationship is still not widely understood. By directing more research towards it, some interesting links and possibilities for therapeutical interventions might arise. Therefore, it is interesting to study the impact energy stress has on cancer cells, and try to unveil the mechanisms between LKB1, a well-known tumour suppressor, AMPK, an energy sensor, and PRC2, which may play a role downstream of these.

Hypothesis



Picture 1. Graphical hypothesis.

The hypothesis of the thesis is based on the possible mechanism for PRC2-mediated gene activation by AMPK during energy stress, described in the 2018 paper by Wan et al. The hypothesis is that energy stress activates AMPK, which then phosphorylates EZH2, inhibiting PRC2 and releasing its target genes from repression. However, the EZH2 T311A mutant cannot be phosphorylated by AMPK, and thus will leave PRC2 active in all energetic conditions. Therefore, cells with mutant EZH2 will have more H3K27me3 during energy stress than their counterparts with wild type EZH2 proteins. The hypothesis therefore predicts that cells with a wild type EZH2 and a mutant EZH2 will behave differently under energy stress, and this can be observed from histone methylation patterns and gene expression levels.

This hypothesis of PRC2 being downstream of AMPK would also bring another point of view into mechanisms non-small cell lung cancer. Inactivation mutations in LKB1 are common in NSCLC, and they lead to AMPK inactivation, which in turn activates PRC2. With an abnormal methylation landscape, downstream tumour and differentiation suppressors lead to worse non-small cell lung cancer. Mutations in LKB1, AMPK and PRC2/EZH2 could therefore have similar effects on lung cancer tumorigenesis through this pathway.

Experimental design

The experimental design makes use of overexpression plasmids EZH2 wild type, EZH2 T311A mutant and GFP. These plasmids are used to make stable overexpression cell lines that can then be treated with 2-deoxyglucose to impose energy stress and study the epigenetic effects. The hypothesis was tested by using Western blotting to detect histone methylation patterns as well as the phosphorylation statuses of AMPK and its known target ACC, and RT-qPCR was used to measure the expression levels of selected target genes *SOX2*, *ATF3*, *HES1*, *PIM1* and *KLF4* in each situation. The results were compared between wild type EZH2 and mutant EZH2 under energy stress conditions.

Experimental hypothesis

The experimental hypothesis of the thesis is that the overexpression cell lines produce PRC2 complexes containing the overexpressed EZH2 protein. The EZH2 T311A overexpression cell lines still have endogenous wild-type EZH2, but the assumption is that the mutant EZH2 proteins will be used in enough PRC2 complexes to see a change in histone modification and gene expression levels. According to the proposed mechanism, the EZH2 T311A mutant cells would behave more similarly when comparing energy stress and normal glucose conditions, whereas wild-type EZH2 overexpression cells and GFP cells would have differences between the two energetic conditions. As for the results, the hypothesis suggests that a significant difference in gene activation of AMPK dependent genes will be observed during glucose deprivation when comparing EZH2 wild type and mutant overexpression cell lines.

Materials and methods

Cell culture

In normal growing conditions, cells were grown in basal media supplemented with 10% foetal bovine serum (FBS; Gibco #10270-106), 2mM L-glutamine, penicillin (100U/ml) and streptomycin (100U/ml). When passaging, the cells were detached from plates using Trypsin-EDTA (0.05%, Gibco #25300054). PBS was used to rinse the cells. The condition in the cell culture incubator were 37°C and 5% CO_2.

H358 cells are an immortalized cancer cell line derived from a minimally invasive lung adenocarcinoma (ATCC website). They were cultured in RPMI 1640 (without L-glutamine, Lonza #12-167F) and above-mentioned supplements.

Cell lines 2352.4 and 2352.9 are immortalized mouse embryonic fibroblasts (MEF), obtained from transgenic mice in our laboratory. In these cell lines, all AMPKα alleles can be conditionally deleted; 2352.4 is a homozygote with both alleles floxed (*Prkaa1* $^{flox/flox}$*; Prkaa2* $^{flox/flox}$) and 2352.9 is a heterozygote (*Prkaa1* $^{+/flox}$*; Prkaa2* $^{+/flox}$). These MEFs were cultured in Dulbecco's Minimum Essential Medium (DMEM), (M5650-500ML; SIGMA Life Science) and above-mentioned supplements.

Plasmids

The EZH2 wild type, EZH2 mutant are in N-terminal p-Flag CMV-2 expression vectors. The plasmids contain an ampicillin resistance gene, a flag tag, and a multiple cloning site. EZH2 wild type and EZH2 T311A mutant plasmids were a gift from Li Xin Wan from Moffit Cancer Centre (Wan et al. 2018). In our laboratory, EZH2 wt and T311A mutant ORFs were cloned into pLenti lentiviral vectors. A GFP plasmid was used as control.

Minipreps and midipreps of plasmids were prepared according to the protocol of the Macherey-Nagel NucleoSpin plasmid DNA purification kit (Macherey-Nagel website).

Virus transfection

pLenti6.3-V5 overexpression lentiviruses were produced by transfecting HEK293FT cells with viral packaging and overexpression vectors. On day one, 6,000,000 cells were passaged on each 10cm plate, using DMEM without antibiotics (penicillin or streptomycin). The transfection was done on day two. Viral packaging vectors Delta8.9 and pLP-VSVG were co-transfected with one of the overexpression lentiviral vectors, pLenti6.3-emGFP, pLenti6.3-EZH2 wt or pLenti6.3-EZH2 T311A. The transfections were performed with Lipofectamine 2000 using manufacturer's instructions. After 4 hours, the transfection media was replaced with fresh DMEM + FBS + L-glutamine. The cells were incubated for 48 hours, and the virus supernatant was collected and passed through a 0.4um filter on day four. The viruses were stored in -70°C.

Lentiviral transduction

A 6-well plate passaged a few days prior was transduced with pLenti viruses EZH2 wt, EZH2 T311A and GFP. Two control wells were left without virus. The transduction media was prepared using 1ml of virus media from -70°C, 1ml of RPMI 1640 + all supplements + Polybrene infection/transfection reagent (Millipore TR-1003-G) 8ug/ml in final concentration. 2ml of transduction media were used per well. The next day, the media was changed to RPMI 1640 + all supplements. The day after this, the selection media containing 5ug/ml blasticidin was changed to all wells except the no virus no blasticidin control. From here on, the blasticidin selection was kept on the overexpression cell lines at all times. The overexpression cell lines were expanded and then frozen for upcoming experiments, and the control wells with blasticidin but no virus were monitored until dead.

Western Blot

Depending on the proteins to be detected, acrylamide gels were either cast by hand or precast gradient gels were used. For the detection of ACC, AMPK, and their phosphorylation status, 7% and 10% gels were used. For the detection of H3 and H3K27me3, 4-20% precast gels (Mini-PROTEAN TGX Gels, 4-20%, 10-well comb, 50ul, BioRad #456-1094) were used. Vinculin or GAPDH were detected as loading controls on each blot.

The samples were prepared according to their protein concentration, loading the same amount of protein into each well. The optimal amount of protein was 20ug in 20ul, but it was decreased when the concentration of the initial sample was not high enough. 20% of Laemmli loading dye was added, and the samples were boiled at 98°C for 5min before pipetting them into the gels. SDS-PAGE gels were run at 50V for 30min, then at 200V until the end of the gel was reached.

After the gel was run, the proteins were blotted with the TurboBlotter machine, using the Trans-Blot Turbo Transfer Pack (BioRad. #1704159). The gels were put onto membranes, and the machine was run for 10min with the BioRad protocol for midi gels. After the run was completed, the membrane was stained with Ponceau S dye (Sigma, P-7170) for 5 minutes to check for an even protein loading, then cut according to the ladder and visible proteins. The membrane was then blocked in 5% milk powder/BSA in 0.1% TBST (TBS, 0.1% Tween-20) for 1h. Milk powder was used for H3 and H3K27me3 and BSA for blots that included phosphorylated proteins (ACC, AMPK, p-ACC

and p-AMPK). After 1h of blocking, the membranes were put into primary antibodies, in rotation in the cold room overnight. See Table 1. for all antibodies.

On the second day of the Western blot protocol, the membranes were washed 3 times for 10min in TBST. The membranes were incubated with secondary antibody dilutions (see antibody table) for 1h in cold room rotation. The membranes were again washed 3 times for 10min in TBST, then imaged. The detection of the proteins was performed with Super Signal West Femto Maximum Sensitivity Substrate kit (Thermo Scientific, #34095), using 2ml of the diluted ECL reagents per membrane. 10% of Super Signal Femto reagents were diluted to 90% of TBS to get the working reagent. This was applied to the membrane, excess was poured off, and the membrane was imaged first with the chemiluminescence setting using ChemiDoc MP Imaging System (Bio-Rad), up to 60 seconds for strong signal intensity and up to 10 minutes for weak intensity. The pictures were then processed on Microsoft PowerPoint.

2-DG treatment

6-well plates passaged at least one day before were used for the 2-DG treatment. Treatment medias were prepared as follows: No glucose DMEM + dialysed FBS + L-glutamine + penicillin-streptomycin as basal media. When indicated, 2-DG in powder form was added to 15mM final concentration, and 1.11 M solution of glucose to get 25 mM final concentration. The old media was taken off of the 6-well plates, the wells were rinsed with PBS and 2ml of the treatment media was added to each well. The plates were treated 20 minutes apart to allow the collection of both protein and RNA at exactly 4h of the start of the treatment.

Protein collection and preparation

Protein samples were collected by aspirating the media from the wells, rinsing once with PBS, then pipetting 200ul of boiling hot LSB into each well. The wells were scraped with a cell scraper, then the samples were collected into Eppendorf tubes. The samples were boiled at 98°C for 5 minutes, then passed through a 25G needle 10 times to shear the DNA. Protein samples were stored in -70°C.

DC-assay

To measure protein concentration, a Detergent-Compatible assay (Bio Rad) was done. Standard protein samples made from bovine serum albumin (BSA) and dissolved in LSB in the following concentrations: 0; 0,25; 0,5; 0,75; 1; 1,5; 2 mg / ml. Duplicates of both standard samples and samples to be tested were pipetted onto a 96-well plate. The DC assay was done according to the protocol from BioRad (BioRad website).

RNA collection and extraction and measurement of RNA concentration

RNA was collected using the Trizol protocol. The media was taken out of the cell culture wells, then Trizol reagent was added 1 ml per 6-well plate well. The wells were incubated for 5 min in room temperature, then collected into Eppendorf tubes. 200 ul of chloroform was added, the tubes were shaken and incubated 2 – 3 min, then centrifuged at 13 000 rpm for 15 min in 4 ˚C. The upper phase was taken into a new Eppendorf tube, and 500 ul of chloroform : isoamylalcohol (24:1) mixture was added. The mixture was centrifuged at 13 000 rpm 3 min, and the water phase was again taken into a new tube. 1 ul glycogen and 500 ul isopropanol were added, and the mixture was incubated for 20 min on ice or overnight in -20 ˚C. The samples were centrifuged 13 000 rpm for 30 min at 9 ˚C, after which the tubes were taken on ice. The supernatant was removed, and the pellet was washed with 1 ml of 70 % ethanol and centrifuged for 5 min. The supernatant was removed, and the ethanol was left to evaporate with the lid open for about 10 min. The pellet was dissolved into 30 – 50 ul milliQ water by putting it into 65 ˚C, then on ice. The RNA samples were stored in -70 ˚C (Thermofisher website).

RNA concentration was measured with the NanoDrop device.

RT-qPCR

cDNA was made from RNA samples by RT-PCR with Taqman Multiscribe reverse transcriptase reagents kit (Applied biosystems Life technologies; #N8080234). Samples were prepared to contain 400ng of RNA in 7.6ul of milliQ water, and one noRT sample was added as a control. 0.75ul RQ1 DNase Promega and 0,9ul 10x were added to each sample and they were incubated at 37°C for 30min. 0,9ul Stop solution was added, followed by a 10min incubation at 65°C. Samples were put on ice, and 2ul 10X RT buffer, 0.3ul MgCl2 25mM, 4ul dNTP mix 2.5mM, 1ul Random primers 50uM,

0.5ul RNasin and 1,5ul milliQ water were added to each tube. 0.5ul MultiScribe reverse transcriptase was added to all samples except the noRT sample. The RT reaction was done as follows: 25°C for 10min, 48°C for 30min, 95°C for 5min and lastly 15°C. The cDNA was stored in +4°C.

For qPCR, the cDNA was diluted to get 10ng of cDNA in 1ul. qPCR was run in 96-well plates, and each well contained 1ul of cDNA, 0.8ul of primer mix with 5uM both forward and reverse primers, 10ul of KAPA SYBR FAST Universal 2X qPCR Master Mix (Sigma-Aldrich # KK4618) and 8,2ul of MilliQ water. The qPCR reaction was run with the StepOnePlus machine, using the StepOnePlus fast cycling protocol with a 3-minute initial denaturation step.

After the run, the qPCR results were analysed on Microsoft Excel.

Antibodies

Name of antibody	**Host**	**Information**	**Dilution**	**Diluted in**	**Use**
H3	Rabbit	Abcam Ab1791	1:10 000	5% milk	Primary
H3K27me3	Rabbit	Millipore 07-449	1:1 000	5% milk	Primary
AMPK	Rabbit	CST#2532	1:500	5% BSA	Primary
p-AMPK	Rabbit	CST#2535	1:500	5% BSA	Primary
ACC	Rabbit	CST36662	1:250	5% BSA	Primary
p-ACC	Rabbit	CST#3661	1:500	5% BSA	Primary
Vinculin	Mouse	sigma V9131	1:30 000	5% milk	Primary
GAPDH	Rabbit	14C10 CST#2118	1:9 000	5% BSA	Primary
V5	Mouse	Invitrogen/Novex R96025, 46-0705	1:1 000	5% BSA	Primary
Rabbit anti Mouse HRP	Rabbit	Millipore AP106P	1:5 000	5% milk / BSA	Secondary
Goat anti Rabbit HRP	Goat	Millipore AP132P	1:5 000	5% milk / BSA	Secondary

Table 1. Antibodies

Results

H358

H358 is a cancer cell line derived from a minimally invasive lung adenocarcinoma (ATCC website). It was chosen to study the AMPK-PRC2 pathway hypothesis in NSCLC cells. H358 was also chosen because of its LKB1 wild type status, a prerequisite for functional AMPK signalling. In addition, LKB1 is often mutated in lung cancer, suggesting that LKB1 downstream substrates may play a role in carcinogenesis (Ji et al. 2007).

H358 cells were transfected with pLenti plasmids EZH2 wild type (wt), EZH2 T311A mutant or GFP, and selected with blasticidin to obtain stable overexpression cell lines. The overexpression was confirmed with imaging of the GFP cell line and Western blot using V5 antibody for EZH2 wt and EZH2 mutant cell lines. Each cell line was then treated with 2-DG or glucose-containing media for 4 hours, and RNA and protein were collected and analysed with RT-qPCR and Western blot, respectively. The H3K27me3 signal on whole cell level was also assessed on Western blot, and total histone 3 used to monitor histone levels.

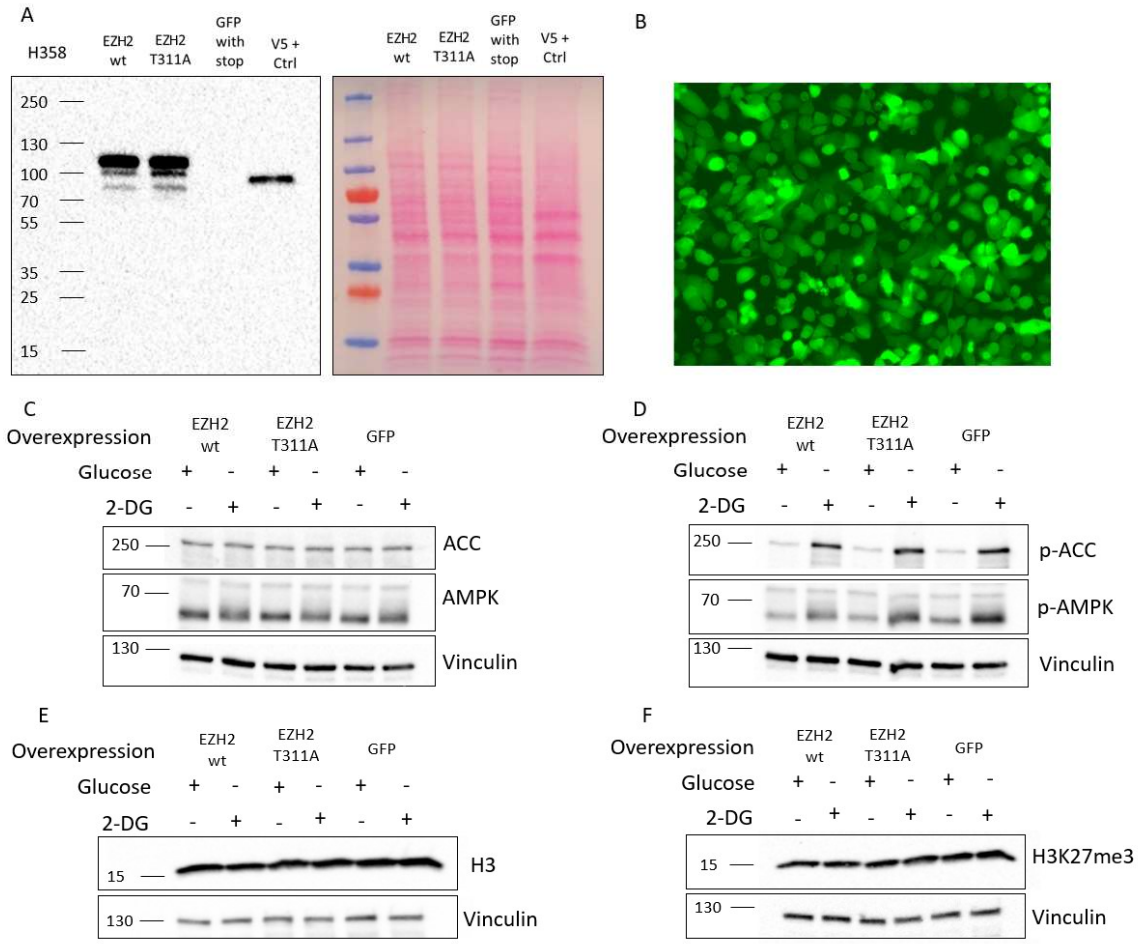


Figure 1. A) Western blot showing the overexpression of V5 flag tag in the overexpression plasmid. The control sample consisted of lysate from a successful V5-tagged overexpression experiment and was included to control antibody function. The GFP plasmid is not V5 tagged due to stop codon preceding the C-terminal tag. Ponceau S staining is used as a loading control. EZH2 protein size is 85kDa and GFP protein size is 28kDa. B) Microscope image showing the expression of GFP in the H358 cell line transfected with the GFP plasmid. Image taken with the EVOS microscope. C) Western blot for total ACC and AMPK. D) Western blot for phosphorylated ACC and AMPK. E) Western blot with the H358 overexpression cell lines EZH2 wt, EZH2 T311A and GFP each treated with 25mM glucose or 15mM 2-DG, detecting the total histone 3 in the cells. F) Western blot detecting the trimethylated lysine 27 of histone 3 in the different cell lines and treatments.

The activity of AMPK was tested with Western blots using the antibodies against AMPK, ACC, and the phosphorylated forms p-AMPK and p-ACC. The blots showed that the total amount of AMPK and ACC stayed the same between the samples, but the phosphorylation status changed with energy stress. ACC was used to confirm normal AMPK activity, as it is a well-known phosphorylation target of AMPK (Dyck et al. 2001), and the phosphorylation of ACC under energy stress conditions was indeed very evident from the Western blots. AMPK also showed an increase in phosphorylation under energy stress with each cell line, which was as expected. The differences between the cell lines are in EZH2, and PRC2 is downstream of AMPK. Therefore, the activity of AMPK was not changed by the overexpression of the EZH2 and GFP plasmids.

The total histone 3 Western blots were used to confirm that the amount of histone did not change between each sample. The H3K27me3 blots showed no significant difference in histone methylation between the samples. The hypothesis was that the methylation status of EZH2 wt and GFP cell lines would be lower during energy stress than with glucose, as energy stress activates AMPK and the PRC2 complex is unable to methylate H3K27. This is however not seen in the Western blots.

The RNA collected from the same experiments as proteins was used to synthesise cDNA with reverse transcription, followed by quantitative PCR.

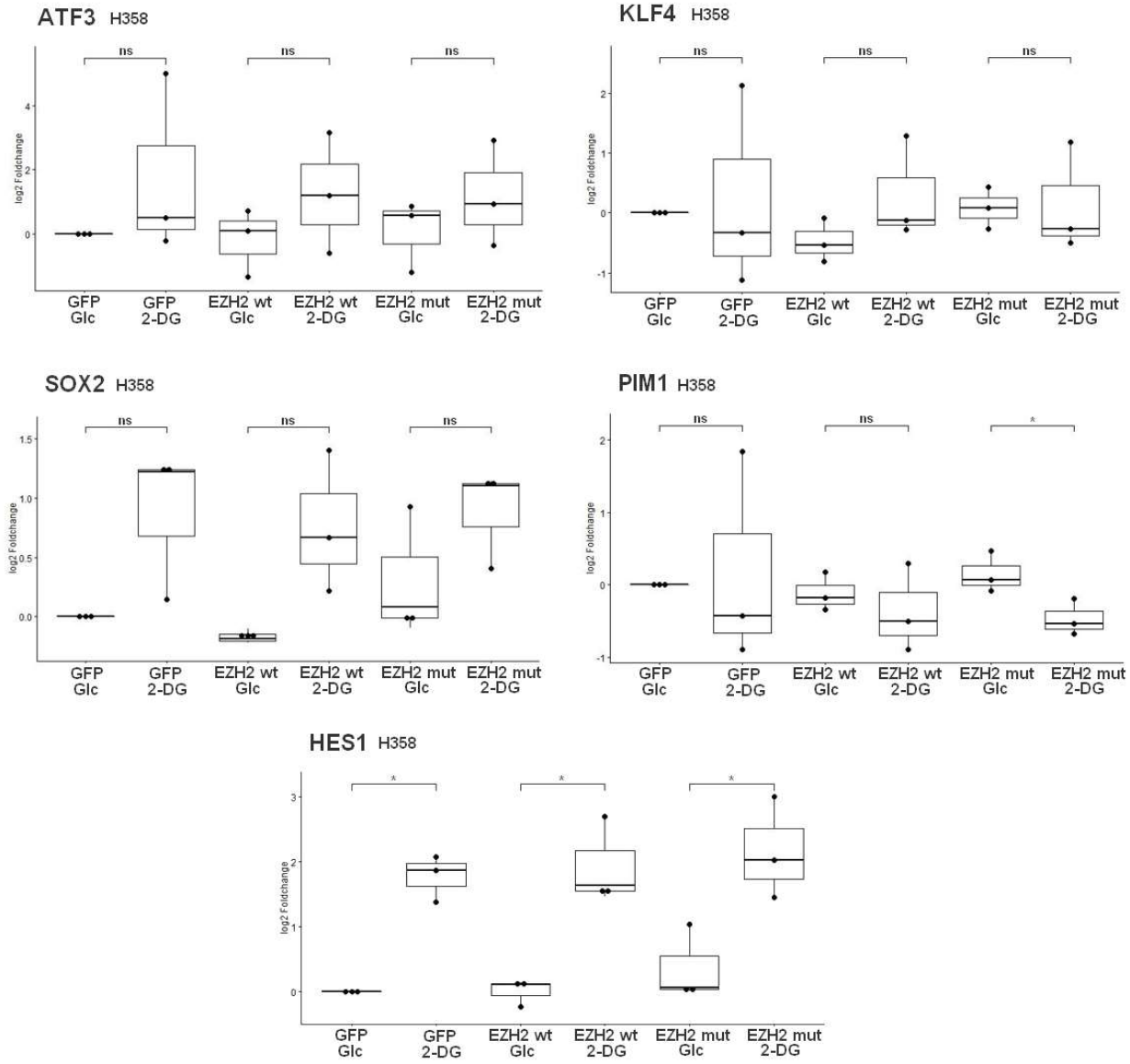


Figure 2. Gene expression levels as log2 fold changes from qPCR. Results for each sample were normalised to *OAZ1*. All results are compared to the glucose treated GFP sample. The results were obtained from three independent experiments. The plots were drawn with R and p-values were calculated with t-test. p-values are indicated as "ns" (non-significant) if over 0.05, * if P is less or equal to 0.05, ** if P is less or equal to 0.01 and *** if P is less or equal to 0.001.

ATF3, *PIM1*, *KLF4* and *SOX2* show non-significant changes between glucose and 2-DG treated samples. There is a lot of variation between replicates and this prevents reaching the significance cutoff. Therefore, these genes do not seem to be reproducibly 2-DG responsive in H358 cells in these

experimental settings. *HES1* gives statistically significant induction in 2-DG samples, but it is not EZH2 dependent, as the EZH2 T311A mutant cell line behaves in a similar way as the other overexpression cell lines. This would indicate that the mechanism by which *HES1* is induced in response to 2-DG would be other than through the T311 phosphorylation site.

Mouse Embryonic Fibroblasts

To test the hypothesis in different cells, mouse embryonic fibroblasts (MEFs) were used to repeat the experiments. The cell line used was 2352.4, with both AMPKα alleles floxed (*Prkaa1* $^{flox/flox}$*; Prkaa2* $^{flox/flox}$). Stable overexpression cell lines were made with the same EZH2 wt, EZH2 mutant and GFP plasmids as for H358, however, lentiviral particles were first produced and the plasmids were incorporated into the cells with lentiviral transduction, as these cells were not amenable to lipid-mediated transfection. The overexpression was confirmed by microscope for GFP and by EZH2 Western blot for EZH2 wt and EZH2 mutant. The overexpression of the wild type EZH2 was a little stronger than the mutant EZH2, but both still stronger than endogenous EZH2 production.

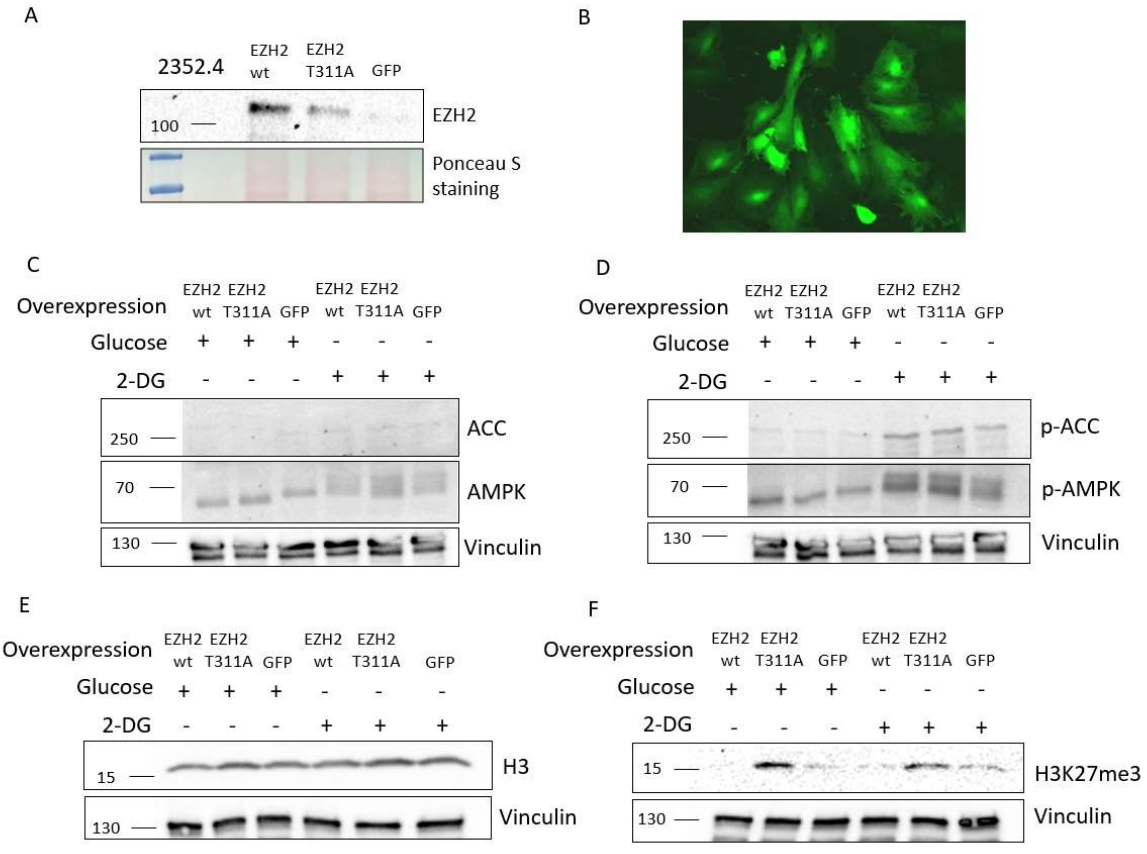


Figure 3. A) Western blot showing the overexpression of the 2352.4 overexpression cell lines. The antibody EZH2 detects both the wild type and T311A mutant EZH2s. Ponceau S staining is used as a loading control. B) Microscope image showing the expression of GFP in the MEF 2352.4 cell line containing the GFP plasmid. Image taken with the EVOS microscope. C) Western blot for total ACC and AMPK. D) Western blot for phosphorylated ACC and AMPK. E) Western blot with the MEF 2352.4 overexpression cell lines EZH2 wt, EZH2 T311A and GFP each treated with 25mM glucose or 15mM 2-DG, detecting the total histone 3 in the cells. F) Western blot detecting the trimethylated lysine 27 of histone 3 in the different cell lines and treatments.

After treating the overexpression cell lines with 2-DG or glucose, RNA and protein samples were collected. Protein samples were used for Western blotting, and antibodies were used to detect the effects on protein phosphorylation. ACC and AMPK both show slightly stronger signal with 2-DG when comparing phosphorylation statuses (Fig. 3). This demonstrates that AMPK is activated by the 2-DG treatment. ACC is a known LKB1 target (Dyck et al. 2001), and it is clearly phosphorylated during energy stress. There is no difference between the cell lines, and the mutation in EZH2 does not have an effect on the phosphorylation of ACC and AMPK.

From the same samples, the H3K27me3 methylation status in whole cell lysate was investigated. The results show that the histone methylation status changes across the samples. The EZH2 T311A mutant samples have the strongest methylation signal, and EZH2 wt the weakest. EZH2 wt shows a very weak signal with both glucose and 2-DG, but EZH2 T311A has a slightly stronger signal with glucose than with 2-DG. According to the hypothesis, the methylation should decrease with energy stress in EZH2 wt and GFP but decrease less in EZH2 T311A. The Western blot does not support this hypothesis very clearly, as the basal levels of H3K27me3 are very low to start with in wt EZH2 overexpressing cells.

To test gene expression levels, the collected RNA was used to synthesise cDNA in with reverse transcription. The cDNA was then used to run qPCR, using primers for the genes *Klf4*, *Atf3*, *Pim1*, *Hes1* and *Sox2*. The results were normalised to *Oaz1*. The 2-DG samples were then compared to the GFP glucose sample. Results show the gene expression fold changes for each overexpression cell line in glucose conditions and 2-DG conditions. The hypothesis was that expression of target genes in EZH2 T311A would be less induced than in EZH2 wt, which has the PRC2 complexes that are able to respond to energy stress and release target genes from repression. *Atf3*, *Klf4* and *Pim1* responded in this way, showing more induction in EZH2 wt than in EZH2 mutant and GFP.

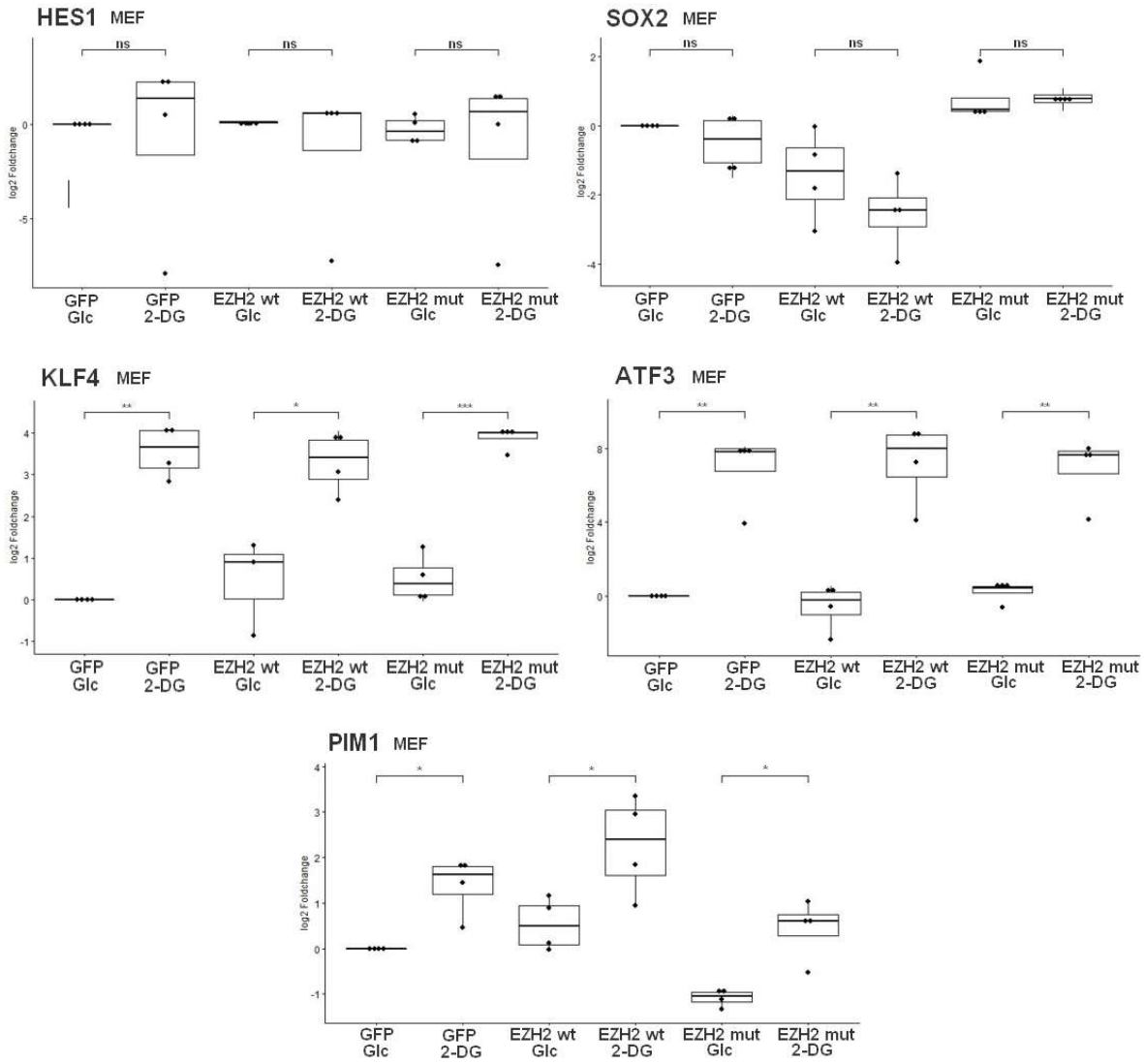


Figure 4. qPCR results from RNA expression after 2-DG or glucose treatment in MEF overexpression cell lines. The values have been normalised to the housekeeping gene *Oaz1* and compared to the glucose GFP sample. The results were obtained from 3 independent experiments, one of them with biological replicates. The plots were drawn with R and p-values were calculated with t-test. p-values are indicated as "ns" (non-significant) if over 0.05, * if P is less or equal to 0.05, ** if P is less or equal to 0.01 and *** if P is less or equal to 0.001.

In *Hes1* and *Sox2*, induction is not statistically significant, thus no reproducible gene expression changes can be detected. Therefore, 2-DG does not activate *Hes1* and *Sox2* in MEFs in this experimental setting. *Klf4* and *Atf3* show statistically significant induction with 2-DG but show very

similar patterns with all overexpression plasmids. This suggests that they are 2-DG responsive possibly through another pathway than EZH2. *Pim1* shows statistically significant induction with 2-DG, and the cell line overexpressing the mutant EZH2 responds differently from GFP and EZH2 wild type cell lines. In normal glucose conditions, the EZH2 T311A cell line has downregulated *Pim1*. The PRC2 complex carrying the mutant EZH2 cannot be phosphorylated and is thus constitutively active, so the genes that are PRC2 dependent are downregulated in comparison to wild type EZH2 containing cells. *Pim1* is likely 2-DG responsive through the T311 phosphorylation site.

Discussion

Lung cancer is among the most common cancers, but still has the worst prognosis. More people die of lung cancer than of breast, prostate, colorectal cancer, and leukaemia combined (Siegel et al. 2019). There is an evident need for more understanding of the mechanisms behind lung cancer.

PRC2 and its catalytic subunit EZH2 have been found to be overexpressed in non-small cell lung cancer, and mutations in their proposed upstream kinases LKB1 and AMPK are also tightly related to NSCLC. Therefore, the aim of this thesis was to test the proposed mechanism for PRC2 and AMPK during energy stress described by Wan et al. 2018, as well as to find potential targets for PRC2. The results suggest that *Pim1* is a target of EZH2 in mouse embryonic fibroblasts and responds to 2-DG stimulation through the T311 site. The results also suggest that *HES1* in H358 cells and *Klf4* and *Atf3* in MEFs are 2-DG responsive, however, not through the T311 site.

The study settings allowed to validate that the use of 2-DG put the cells into a state of energy stress. 2-deoxyglucose is a glucose analog that lacks one hydroxyl group. It is taken into the cell through the same glucose transporters as glucose but is then converted to 2-deoxyglucose-6-phosphate (2DG-6P), which cannot be used in the next step of glycolysis to produce ATP. In addition, 2DG-6P accumulates into the cell, which inhibits the glycolysis enzyme hexokinase. Therefore, 2-DG establishes energy stress in two ways – by competing with glucose in glycolysis, without the production of ATP and by inhibiting the entry of glucose into glycolysis (Schmidt et al. 2020). In our study setting, cells treated with 2-DG also have no glucose in their media, making sure there is no ATP produced through glycolysis. The increased AMP/ATP ratio activates AMPK, which regulates the expression of genes involved in many pathways. The transcriptional changes that happen in the cells are most likely related to energy metabolism, switching from anabolic pathways to catabolic ones.

In this study, cells treated with 2-DG had elevated AMPK phosphorylation, implying that the LKB1/AMPK energy sensing axis worked in these settings. The phosphorylation status of ACC was also elevated, suggesting that activated AMPK was phosphorylating its downstream targets, as expected. This most likely has changed the transcription of other genes, as AMPK regulates glucose, lipid and protein metabolism when activated. If PRC2 is a direct target of AMPK during energy stress, it is probable that also EZH2 was phosphorylated in these conditions. There are many possible reasons why this would not have been visible in the histone methylation statuses.

Assuming that the hypothesis of AMPK and PRC2 crosstalk is correct, energy stress conditions should change the methylation patterns of target gene promoters. However, no significant changes in methylation status were observed in Western blots detecting H3K27me3. One possible reason is that the samples used were whole cell lysate, and the H3K27me3 detected is from all areas of the genome, including regions that are not related to energy stress gene expression. H3K27me3 is located both at specific peaks and is spread over an entire locus, and three different enrichment profiles have been observed for H3K27me3. It is found on repressed genes, but also highly expressed ones, and around the transcription start site on bivalent genes, where it co-exists with H3K4me3 (Young et al. 2011). The number of target genes that experience a change in methylation patterns due to energy stress might be so small, that their part in overall H3K27me3 is not detectable.

As to why only one of the studied target genes followed the hypothesis, the reason might be in the experimental settings. The results rely on the assumption that the overexpressed mutant EZH2 protein is incorporated into enough PRC2 complexes to have an impact big enough for it to affect histone methylation statuses and gene expression levels. These research settings do not prove that the mutant EZH2 gets incorporated into PRC2 complexes, although the results about *Pim1* in MEFs would suggest that it does.

The cell line overexpressing wild type EZH2 works as a control for what effects an overexpression of EZH2 could have. EZH2 is often overexpressed in cancers and correlates with poor prognosis, NSCLC included (Takawa et al. 2011), which leads to the question whether it could promote an increased activity of PRC2. However, no evidence was found in literature claiming that the overexpression of EZH2 would promote the overexpression of other PRC2 subunits. Rather, the overexpression of EZH2 is not considered to affect the methylation status of H3K27, as it requires the overexpression of other subunits as well (Conway et al. 2015). In addition, the results from *Pim1* in MEFs would suggest that EZH2 is not a limiting factor. Comparing to the GFP overexpressing cell line treated with

glucose, the expression of *Pim1* is not repressed in cells overexpressing EZH2 in glucose conditions. Furthermore, there is some evidence showing that the overexpression of EZH2 that is observed in multiple cancers correlates with other proliferation markers and could be a product of high proliferation of cancer cells, rather than a reason for it (Wassef et al. 2017). Therefore, overexpressing EZH2 wt is not expected change to the activity of PRC2 in the cell, but a difference in gene expression and methylation patterns is expected be seen with the expression of EZH2 T311A. To see a difference in the results, the EZH2 mutant should occupy enough PRC2 complexes to make the number of PRC2 complexes with endogenous wild type EZH2 significantly lower than in EZH2 wt and GFP overexpressing cells.

Although the overexpression of EZH2 is not expected to change the activity of PRC2, the overexpression of EZH2 could, however, affect the cells in other ways. There is some evidence of EZH2 acting as an activator independently from the PRC2 complex (Xu et al. 2012; Lee et al. 2011; Shi et al. 2007). However, most of EZH2's activity is connected to the PRC2 complex (Wassef et al. 2019), and both histone 3 lysine 27 and the target genes studied in this thesis are linked to the repressive mark catalysed by the PRC2 complex, implying that solo EZH2 activity should not have an impact on the results. Nonetheless, there may be consequences for overexpressing EZH2 that are not taken into account in this study or even not known yet, such as the finding that overexpression of EZH2 promotes the formation of an previously unknown Polycomb complex, PRC4 (Kuzmichev et al. 2005).

What must also be taken into account in these study settings is that although multiple repetitions of the experiments were done, there was only one of each overexpression cell lines. This raises the question of whether the properties seen in the results are due to the protein function or a characteristic of the specific pool of cells. Mutations could have happened during the process of making the overexpression cell lines and growing of the cells. However, due to the slow growth rate of the cells, it was not possible to shorten the time that they were in culture. The cells were not used past passage 18 counting from transduction.

In conclusion, the results suggest that *Pim1* could be a target gene of PRC2 in mouse embryonic fibroblasts, and that its activation during energy stress happens via the EZH2 T311 site. However, the overexpression of EZH2 wild type and mutant may not be enough to test the hypothesis, as methylation statuses and other target genes did not show significant results.

Future directions

More research would be needed to validate the hypothesis stated in this thesis. An interesting follow-up project would be to use MEF cell lines 2352.4 and 2352.9 and to delete the floxed AMPKα alleles. The deletion of AMPK would allow to study how the cell reacts to energy stress when there is no AMPK. This way the hypothesis of the mechanism could be tested further, to see whether PRC2 is only activated by AMPK during energy stress.

In addition, studying the amounts of PRC2 subunits as free proteins and bound to other subunits could reveal information about the dynamics of the PRC2 complex. Identifying the possible limiting factor to the construction of more PRC2 complexes and finding out whether an overexpression of specific subunits trigger changes in the expression of other PRC2 subunits could increase the information about this epigenetic mechanism. Although EZH2 overexpression has been detected in multiple different cancers, the effect this overexpression has on the number of functional PRC2 complexes and their activity is still not well understood.

Acknowledgements

First and foremost, I am deeply grateful to my supervisor Elina Niemelä, who taught me everything I know and guided me in lab work, lab life and the writing process. Thank you for always being patient and helping me navigate in the world of science.

I would like to express my gratitude to Tomi Mäkelä for the opportunity to perform this thesis work in his laboratory and for taking the time to guide me in my project. I would also like to remember my co-workers who I could always count on for solving problems and having lunch and coffee breaks with. In addition, I would like to thank TRANSMED director Tiina Immonen, who guided and helped us all through our studies.

Furthermore, I'd like to thank my fellow TRANSMEDs, especially Anh, Veera, Neja and Felicia, everything was much more fun and motivating because of you. Special thanks to my parents and sister for always supporting me, believing in me, and showing interest in this topic, despite being very far from the field. Finally, I'd like to thank my statistically significant other Erik for being the best home office co-worker and keeping me sane for the whole year of studying and writing this thesis from home. Thank you for always being by my side.

References

https://www.lgcstandards-atcc.org/Products/All/CRL-5807 ATCC website, accessed 7.4.2020

https://www.mn-net.com/media/pdf/45/51/02/Instruction-NucleoSpin-Plasmid.pdf Macherey-Nagel website, accessed 7.4.2020

http://www.bio-rad.com/webroot/web/pdf/lsr/literature/LIT448.pdf (BioRad website, accessed 7.4.2020)

https://assets.thermofisher.com/TFS-Assets/LSG/manuals/trizol_reagent.pdf (Thermofisher website, accessed 7.4.2020)

Albert, M., & Helin, K. (2010, April). Histone methyltransferases in cancer. In *Seminars in cell & developmental biology* (Vol. 21, No. 2, pp. 209-220). Academic Press.

Alessi, D. R., Sakamoto, K., & Bayascas, J. R. (2006). LKB1-dependent signaling pathways. *Annu. Rev. Biochem*., 75, 137-163.

Bass, A. J., Watanabe, H., Mermel, C. H., Yu, S., Perner, S., Verhaak, R. G., ... & Meyerson, M. (2009). SOX2 is an amplified lineage-survival oncogene in lung and esophageal squamous cell carcinomas. *Nature genetics*, 41(11), 1238-1242.

Behrens, C., Solis, L. M., Lin, H., Yuan, P., Tang, X., Kadara, H., ... & Wistuba, I. I. (2013). EZH2 protein expression associates with the early pathogenesis, tumor progression, and prognosis of non–small cell lung carcinoma. *Clinical cancer research*, 19(23), 6556-6565.

Beltran, M., Tavares, M., Justin, N., Khandelwal, G., Ambrose, J., Foster, B. M., ... & Jenner, R. G. (2019). G-tract RNA removes Polycomb repressive complex 2 from genes. *Nature structural & molecular biology*, 26(10), 899-909.

Blanco, R., Iwakawa, R., Tang, M., Kohno, T., Angulo, B., Pio, R., ... & Sanchez-Cespedes, M. (2009). A gene-alteration profile of human lung cancer cell lines. *Human mutation*, 30(8), 1199-1206.

Bowker, S. L., Majumdar, S. R., Veugelers, P., & Johnson, J. A. (2006). Increased cancer-related mortality for patients with type 2 diabetes who use sulfonylureas or insulin. *Diabetes care*, 29(2), 254-258.

Betts, D. R., Stanchescu, R., Niggli, F. K., Cohen, N., Rechavi, G., Amariglio, N., & Trakhtenbrot, L. (2008). SKY reveals a high frequency of unbalanced translocations involving chromosome 6 in t (12; 21)-positive acute lymphoblastic leukemia. *Leukemia research*, 32(1), 39-43.

Carretero, J., Medina, P. P., Pio, R., Montuenga, L. M., & Sanchez-Cespedes, M. (2004). Novel and natural knockout lung cancer cell lines for the LKB1/STK11 tumor suppressor gene. *Oncogene*, 23(22), 4037-4040.

Chen, Y., Huang, Y., Huang, Y., Chen, J., Wang, S., & Zhou, J. (2013). The prognostic value of SOX2 expression in non-small cell lung cancer: a meta-analysis. *PLoS One*, 8(8), e71140.

Cho, Y. G., Song, J. H., Kim, C. J., Nam, S. W., Yoo, N. J., Lee, J. Y., & Park, W. S. (2007). Genetic and epigenetic analysis of the KLF4 gene in gastric cancer. *Apmis*, 115(7), 802-808.

Conway, E., Healy, E., & Bracken, A. P. (2015). PRC2 mediated H3K27 methylations in cellular identity and cancer. *Current opinion in cell biology*, 37, 42-48.

Deb, G., Singh, A. K., & Gupta, S. (2014). EZH2: not EZHY (easy) to deal. *Molecular cancer research*, 12(5), 639-653.

Dimou, A., Dincman, T., Evanno, E., Gemmill, R. M., Roche, J., & Drabkin, H. A. (2017). Epigenetics during EMT in lung cancer: EZH2 as a potential therapeutic target. *Cancer Treatment and Research Communications*, *12*, 40-48.

Dyck, J. R., Kudo, N., Barr, A. J., Davies, S. P., Hardie, D. G., & Lopaschuk, G. D. (1999). Phosphorylation control of cardiac acetyl-CoA carboxylase by cAMP-dependent protein kinase and 5'-AMP activated protein kinase. *European journal of biochemistry*, *262*(1), 184-190.

Escobar, T. M., Kanellopoulou, C., Kugler, D. G., Kilaru, G., Nguyen, C. K., Nagarajan, V., ... & Muljo, S. A. (2014). miR-155 activates cytokine gene expression in Th17 cells by regulating the DNA-binding protein Jarid2 to relieve polycomb-mediated repression. *Immunity*, 40(6), 865-879.

Esposito, L., Conti, D., Ailavajhala, R., Khalil, N., & Giordano, A. (2010). Lung cancer: are we up to the challenge? *Current genomics*, 11(7), 513.

Ezhkova, E., Pasolli, H. A., Parker, J. S., Stokes, N., Su, I. H., Hannon, G., ... & Fuchs, E. (2009). Ezh2 orchestrates gene expression for the stepwise differentiation of tissue-specific stem cells. *Cell*, *136*(6), 1122-1135.

Fadous Khalifé, M. C., Aloulou, N., Jalbout, M., Hadchity, J., Aftimos, G., Paris, F., & Hadchity, E. (2016). Krüppel-like factor 4: A new potential biomarker of lung cancer. *Molecular and clinical oncology*, 5(1), 35-40.

Faubert, B., Boily, G., Izreig, S., Griss, T., Samborska, B., Dong, Z., ... & Mamer, O. A. (2013). AMPK is a negative regulator of the Warburg effect and suppresses tumor growth in vivo. *Cell metabolism*, *17*(1), 113-124.

Garcia, D., & Shaw, R. J. (2017). AMPK: mechanisms of cellular energy sensing and restoration of metabolic balance. *Molecular cell*, 66(6), 789-800.

Ghaleb, A. M., & Yang, V. W. (2017). Krüppel-like factor 4 (KLF4): what we currently know. *Gene*, 611, 27-37.

Goldberg, A. D., Allis, C. D., & Bernstein, E. (2007). Epigenetics: a landscape takes shape. *Cell*, *128*(4), 635-638.

Gowans, G. J., Hawley, S. A., Ross, F. A., & Hardie, D. G. (2013). AMP is a true physiological regulator of AMP-activated protein kinase by both allosteric activation and enhancing net phosphorylation. *Cell metabolism*, 18(4), 556-566.

Greathouse, K. L., Bredfeldt, T., Everitt, J. I., Lin, K., Berry, T., Kannan, K., ... & Walker, C. L. (2012). Environmental estrogens differentially engage the histone methyltransferase EZH2 to increase risk of uterine tumorigenesis. *Molecular Cancer Research*, 10(4), 546-557.

Guo, X., Wang, Z., Lu, C., Hong, W., Wang, G., Xu, Y., ... & Kang, J. (2018). LincRNA-1614 coordinates Sox2/PRC2-mediated repression of developmental genes in pluripotency maintenance. *Journal of molecular cell biology*, 10(2), 118-129.

Hadad, S. M., Baker, L., Quinlan, P. R., Robertson, K. E., Bray, S. E., Thomson, G., ... & Thompson, A. M. (2009). Histological evaluation of AMPK signalling in primary breast cancer. *BMC cancer*, 9(1), 1-9.

Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. *cell*, 100(1), 57-70.

Hardie, D. G. (2015). Molecular pathways: is AMPK a friend or a foe in cancer? *Clinical Cancer Research*, *21*(17), 3836-3840.

Hardie, D. G., Ross, F. A., & Hawley, S. A. (2012). AMPK: a nutrient and energy sensor that maintains energy homeostasis. *Nature reviews Molecular cell biology*, *13*(4), 251-262.

Hawley, S. A., Boudeau, J., Reid, J. L., Mustard, K. J., Udd, L., Mäkelä, T. P., ... & Hardie, D. G. (2003). Complexes between the LKB1 tumor suppressor, STRADα/β and MO25α/β are upstream kinases in the AMP-activated protein kinase cascade. *Journal of biology*, 2(4), 1-16.

Hu, W., Hofstetter, W. L., Li, H., Zhou, Y., He, Y., Pataer, A., ... & Fang, B. (2009). Putative tumor-suppressive function of Krüppel-like factor 4 in primary lung carcinoma. *Clinical Cancer Research*, 15(18), 5688-5695.

Huang, X., Li, X., & Guo, B. (2008). KLF6 induces apoptosis in prostate cancer cells through up-regulation of ATF3. *Journal of biological chemistry*, 283(44), 29795-29801.

Hussain, M., Rao, M., Humphries, A. E., Hong, J. A., Liu, F., Yang, M., ... & Schrump, D. S. (2009). Tobacco smoke induces polycomb-mediated repression of Dickkopf-1 in lung cancer cells. *Cancer research*, *69*(8), 3570-3578.

Inoki, K., Zhu, T., & Guan, K. L. (2003). TSC2 mediates cellular energy response to control cell growth and survival. *Cell*, 115(5), 577-590.

Jan, Y. H., Tsai, H. Y., Yang, C. J., Huang, M. S., Yang, Y. F., Lai, T. C., ... & Hsiao, M. (2012). Adenylate kinase-4 is a marker of poor clinical outcomes that promotes metastasis of lung cancer by downregulating the transcription factor ATF3. *Cancer research*, 72(19), 5119-5129.

Ji, H., Ramsey, M. R., Hayes, D. N., Fan, C., McNamara, K., Kozlowski, P., ... & Liang, M. C. (2007). LKB1 modulates lung cancer differentiation and metastasis. *Nature*, 448(7155), 807-810.

Jin, Y., Tong, D. Y., Tang, L. Y., Chen, J. N., Zhou, J., Feng, Z. Y., & Shao, C. K. (2012). Expressions of osteopontin (OPN), αvβ3 and Pim-1 associated with poor prognosis in non-small cell lung cancer (NSCLC). *Chinese journal of cancer research*, 24(2), 103-108.

Kim, T. M., Yim, S. H., Lee, J. S., Kwon, M. S., Ryu, J. W., Kang, H. M., ... & Chung, Y. J. (2005). Genome-wide screening of genomic alterations and their clinicopathologic implications in non–small cell lung cancers. *Clinical cancer research*, 11(23), 8235-8242.

Kim, Y. H., Liang, H., Liu, X., Lee, J. S., Cho, J. Y., Cheong, J. H., ... & Powis, G. (2012). AMPKα modulation in cancer progression: multilayer integrative analysis of the whole transcriptome in Asian gastric cancer. *Cancer research*, 72(10), 2512-2521.

Kim, W., Youn, H., Kwon, T., Kang, J., Kim, E., Son, B., ... & Youn, B. (2013). PIM1 kinase inhibitors induce radiosensitization in non-small cell lung cancer cells. *Pharmacological research*, 70(1), 90-101.

Kimura, H. (2013). Histone modifications for human epigenome analysis. *Journal of human genetics*, *58*(7), 439-445.

Kleer, C. G., Cao, Q., Varambally, S., Shen, R., Ota, I., Tomlins, S. A., ... & Sabel, M. S. (2003). EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells. *Proceedings of the National Academy of Sciences*, *100*(20), 11606-11611.

Kuzmichev, A., Margueron, R., Vaquero, A., Preissner, T. S., Scher, M., Kirmizis, A., ... & Reinberg, D. (2005). Composition and histone substrates of polycomb repressive group complexes change during cellular differentiation. *Proceedings of the National Academy of Sciences*, 102(6), 1859-1864.

Laugesen, A., Højfeldt, J. W., & Helin, K. (2019). Molecular mechanisms directing PRC2 recruitment and H3K27 methylation. *Molecular cell*, 74(1), 8-18.

Lee, S. T., Li, Z., Wu, Z., Aau, M., Guan, P., Karuturi, R. M., ... & Yu, Q. (2011). Context-specific regulation of NF-κB target gene expression by EZH2 in breast cancers. *Molecular cell*, 43(5), 798-810.

Levine, A. J., & Puzio-Kuter, A. M. (2010). The control of the metabolic switch in cancers by oncogenes and tumor suppressor genes. *Science*, 330(6009), 1340-1344.

Liu, C., Shi, X., Wang, L., Wu, Y., Jin, F., Bai, C., & Song, Y. (2014). SUZ12 is involved in progression of non-small cell lung cancer by promoting cell proliferation and metastasis. *Tumor Biology*, 35(6), 6073-6082.

Liu, M., Li, X., Peng, K. Z., Gao, T., Cui, Y., Ma, N., ... & Hou, G. (2018). Subcellular localization of Klf4 in non-small cell lung cancer and its clinical significance. *Biomedicine & Pharmacotherapy*, 99, 480-485.

Liu, Y. L., Gao, X., Jiang, Y., Zhang, G., Sun, Z. C., Cui, B. B., & Yang, Y. M. (2015). Expression and clinicopathological significance of EED, SUZ12 and EZH2 mRNA in colorectal cancer. *Journal of cancer research and clinical oncology*, 141(4), 661-669.

Liu, Z. H., Dai, X. M., & Du, B. (2015). Hes1: a key role in stemness, metastasis and multidrug resistance. *Cancer biology & therapy*.

Lizcano, J. M., Göransson, O., Toth, R., Deak, M., Morrice, N. A., Boudeau, J., ... & Alessi, D. R. (2004). LKB1 is a master kinase that activates 13 kinases of the AMPK subfamily, including MARK/PAR-1. *The EMBO journal*, 23(4), 833-843.

Luger, K., Mäder, A. W., Richmond, R. K., Sargent, D. F., & Richmond, T. J. (1997). Crystal structure of the nucleosome core particle at 2.8 Å resolution. *Nature*, 389(6648), 251-260.

Margueron, R., & Reinberg, D. (2011). The Polycomb complex PRC2 and its mark in life. *Nature*, *469*(7330), 343-349.

Matsumoto, S., Iwakawa, R., Takahashi, K., Kohno, T., Nakanishi, Y., Matsuno, Y., ... & Yokota, J. (2007). Prevalence and specificity of LKB1 genetic alterations in lung cancers. *Oncogene*, 26(40), 5911-5918.

Mazzio, E. A., & Soliman, K. F. (2012). Basic concepts of epigenetics: impact of environmental signals on gene expression. *Epigenetics*, *7*(2), 119-130.

Minokoshi, Y., Kim, Y. B., Peroni, O. D., Fryer, L. G., Müller, C., Carling, D., & Kahn, B. B. (2002). Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase. *Nature*, 415(6869), 339-343.

Mukhopadhyay, A., Berrett, K. C., Kc, U., Clair, P. M., Pop, S. M., Carr, S. R., ... & Oliver, T. G. (2014). Sox2 cooperates with Lkb1 loss in a mouse model of squamous cell lung cancer. *Cell reports*, 8(1), 40-49.

Nozawa, R. S., Nagao, K., Igami, K. T., Shibata, S., Shirai, N., Nozaki, N., ... & Obuse, C. (2013). Human inactive X chromosome is compacted through a PRC2-independent SMCHD1-HBiX1 pathway. *Nature structural & molecular biology*, 20(5), 566.

Ntziachristos, P., Tsirigos, A., Van Vlierberghe, P., Nedjic, J., Trimarchi, T., Flaherty, M. S., ... & Aifantis, I. (2012). Genetic inactivation of the polycomb repressive complex 2 in T cell acute lymphoblastic leukemia. *Nature medicine*, 18(2), 298-302.

Palomero, T., Lim, W. K., Odom, D. T., Sulis, M. L., Real, P. J., Margolin, A., ... & Ferrando, A. A. (2006). NOTCH1 directly regulates c-MYC and activates a feed-forward-loop transcriptional network promoting leukemic cell growth. *Proceedings of the National Academy of Sciences*, 103(48), 18261-18266.

Pedersen, M. T., & Helin, K. (2010). Histone demethylases in development and disease. *Trends in cell biology*, 20(11), 662-671.

Pelzer, A. E., Bektic, J., Haag, P., Berger, A. P., Pycha, A., Schäfer, G., ... & Klocker, H. (2006). The expression of transcription factor activating transcription factor 3 in the human prostate and its regulation by androgen in prostate cancer. *The Journal of urology*, 175(4), 1517-1522.

Ringrose, L., & Paro, R. (2004). Epigenetic regulation of cellular memory by the Polycomb and Trithorax group proteins. *Annu. Rev. Genet.*, *38*, 413-443.

Ross, F. A., MacKintosh, C., & Hardie, D. G. (2016). AMP-activated protein kinase: a cellular energy sensor that comes in 12 flavours. *The FEBS journal*, 283(16), 2987-3001.

Sanchez-Cespedes, M., Parrella, P., Esteller, M., Nomoto, S., Trink, B., Engles, J. M., ... & Sidransky, D. (2002). Inactivation of LKB1/STK11 is a common event in adenocarcinomas of the lung. *Cancer research*, 62(13), 3659-3662.

Saxena, A., & Carninci, P. (2011). Long non-coding RNA modifies chromatin: epigenetic silencing by long non-coding RNAs. *Bioessays, 33*(11), 830-839.

Schmidt, M. C., & O'Donnell, A. F. (2020). 'Sugarcoating'2-deoxyglucose: mechanisms that suppress its toxic effects. *Current Genetics*, 1-8.

Schuettengruber, B., Chourrout, D., Vervoort, M., Leblanc, B., & Cavalli, G. (2007). Genome regulation by polycomb and trithorax proteins. *Cell*, *128*(4), 735-745.

Sen, G. L., Webster, D. E., Barragan, D. I., Chang, H. Y., & Khavari, P. A. (2008). Control of differentiation in a self-renewing mammalian tissue by the histone demethylase JMJD3. *Genes & development*, 22(14), 1865-1870.

Shah, N., Pang, B., Yeoh, K. G., Thorn, S., Chen, C. S., Lilly, M. B., & Salto-Tellez, M. (2008). Potential roles for the PIM1 kinase in human cancer–a molecular and therapeutic appraisal. *European Journal of Cancer*, 44(15), 2144-2151.

Shaw, R. J. (2006). Glucose metabolism and cancer. *Current opinion in cell biology*, *18*(6), 598-608.

Shaw, R. J., Kosmatka, M., Bardeesy, N., Hurley, R. L., Witters, L. A., DePinho, R. A., & Cantley, L. C. (2004). The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. *Proceedings of the National Academy of Sciences*, *101*(10), 3329-3335.

Shi, B., Liang, J., Yang, X., Wang, Y., Zhao, Y., Wu, H., ... & Shang, Y. (2007). Integration of estrogen and Wnt signaling circuits by the polycomb group protein EZH2 in breast cancer cells. *Molecular and cellular biology*, 27(14), 5105-5119.

Sholl LM, Barletta JA, Yeap BY, Chirieac LR, Hornick JL (2010) Sox2 protein expression is an independent poor prognostic indicator in stage I lung adenocarcinoma. *Am J Surg Pathol* 34: 1193–1198.

Siegel, R. L., Miller, K. D., & Jemal, A. (2019). Cancer statistics, 2019. *CA: a cancer journal for clinicians*, 69(1), 7-34.

Song, X., Lu, F., Liu, R. Y., Lei, Z., Zhao, J., Zhou, Q., & Zhang, H. T. (2012). Association between the ATF3 gene and non-small cell lung cancer. *Thoracic Cancer*, 3(3), 217-223.

Sukumaran, A., Choi, K., & Dasgupta, B. (2020). Insight on transcriptional regulation of the energy sensing AMPK and biosynthetic mTOR pathway genes. *Frontiers in Cell and Developmental Biology,* 8.

Syed, V., Mukherjee, K., Lyons-Weiler, J., Lau, K. M., Mashima, T., Tsuruo, T., & Ho, S. M. (2005). Identification of ATF-3, caveolin-1, DLC-1, and NM23-H2 as putative antitumorigenic, progesterone-regulated genes for ovarian cancer cells by gene profiling. *Oncogene*, 24(10), 1774-1787.

Takawa, M., Masuda, K., Kunizaki, M., Daigo, Y., Takagi, K., Iwai, Y., ... & Hamamoto, R. (2011). Validation of the histone methyltransferase EZH2 as a therapeutic target for various types of human cancer and as a prognostic marker. *Cancer science*, 102(7), 1298-1305.

Thompson, M. R., Xu, D., & Williams, B. R. (2009). ATF3 transcription factor and its emerging roles in immunity and cancer. *Journal of molecular medicine*, 87(11), 1053.

Valdman, A., Fang, X., Pang, S. T., Ekman, P., & Egevad, L. (2004). Pim-1 expression in prostatic intraepithelial neoplasia and human prostate cancer. *The Prostate*, 60(4), 367-371.

Varambally, S., Dhanasekaran, S. M., Zhou, M., Barrette, T. R., Kumar-Sinha, C., Sanda, M. G., ... & Rubin, M. A. (2002). The polycomb group protein EZH2 is involved in progression of prostate cancer. *Nature*, *419*(6907), 624-629.

Velcheti V, Schalper K, Yao X, Cheng H, Kocoglu M, et al. (2013) High SOX2 Levels Predict Better Outcome in Non-Small Cell Lung Carcinomas. *PLoS One* 8: e61427.

Virmani, A. K., Fong, K. M., Kodagoda, D., McIntire, D., Hung, J., Tonk, V., ... & Gazdar, A. F. (1998). Allelotyping demonstrates common and distinct patterns of chromosomal loss in human lung cancer types. Genes, *Chromosomes and Cancer*, 21(4), 308-319.

Wan, L., Xu, K., Wei, Y., Zhang, J., Han, T., Fry, C., ... & Xia, W. (2018). Phosphorylation of EZH2 by AMPK suppresses PRC2 methyltransferase activity and oncogenic function. *Molecular cell*, 69(2), 279-291.

Wang, S. Q., Liu, J., Qin, J., Zhu, Y., Tin, V. P. C., Yam, J. W. P., ... & Xiao, Z. J. (2020). CAMK2A supported tumor initiating cells of lung adenocarcinoma by upregulating SOX2 through EZH2 phosphorylation. *Cell death & disease*, 11(6), 1-13.

Wassef, M., & Margueron, R. (2017). The multiple facets of PRC2 alterations in cancers. *Journal of molecular biology*, 429(13), 1978-1993.

Wassef, M., Luscan, A., Aflaki, S., Zielinski, D., Jansen, P. W., Baymaz, H. I., ... & Margueron, R. (2019). EZH1/2 function mostly within canonical PRC2 and exhibit proliferation-dependent redundancy that shapes mutational signatures in cancer. *Proceedings of the National Academy of Sciences*, 116(13), 6075-6080.

Wu, C. A., Chao, Y., Shiah, S. G., & Lin, W. W. (2013). Nutrient deprivation induces the Warburg effect through ROS/AMPK-dependent activation of pyruvate dehydrogenase kinase. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, 1833(5), 1147-1156.

Xu, K., Wu, Z. J., Groner, A. C., He, H. H., Cai, C., Lis, R. T., ... & Brown, M. (2012). EZH2 oncogenic activity in castration-resistant prostate cancer cells is Polycomb-independent. *Science*, 338(6113), 1465-1469.

Yamauchi, T., Kamon, J., Minokoshi, Y. A., Ito, Y., Waki, H., Uchida, S., ... & Kadowaki, T. (2002). Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nature medicine*, 8(11), 1288-1295.

Yin, X., Dewille, J. W., & Hai, T. (2008). A potential dichotomous role of ATF3, an adaptive-response gene, in cancer development. *Oncogene*, 27(15), 2118-2127.

Young, L. H. (2009). A crystallized view of AMPK activation. *Cell Metabolism*, *10*(1), 5-6.

Young, M. D., Willson, T. A., Wakefield, M. J., Trounson, E., Hilton, D. J., Blewitt, M. E., ... & Majewski, I. J. (2011). ChIP-seq analysis reveals distinct H3K27me3 profiles that correlate with transcriptional activity. *Nucleic acids research*, 39(17), 7415-7427.

Yuan, X., Wu, H., Xu, H., Han, N., Chu, Q., Yu, S., ... & Wu, K. (2015). Meta-analysis reveals the correlation of Notch signaling with non-small cell lung cancer progression and prognosis. *Scientific reports*, 5, 10338.

Zhang, C., Liu, J., Zhang, Y., Luo, C., Zhu, T., Zhang, R., & Yao, R. (2019). LINC01210 accelerates proliferation, invasion and migration in ovarian cancer through epigenetically downregulating KLF4. *Biomedicine & Pharmacotherapy*, 119, 109431.

Zhang, H., Brainson, C. F., Koyama, S., Redig, A. J., Chen, T., Li, S., ... & Lu, G. (2017). Lkb1 inactivation drives lung cancer lineage switching governed by Polycomb Repressive Complex 2. *Nature communications*, 8(1), 1-15.

Zhang, H., Qi, J., Reyes, J. M., Li, L., Rao, P. K., Li, F., ... & Wong, K. K. (2016). Oncogenic deregulation of EZH2 as an opportunity for targeted therapy in lung cancer. *Cancer discovery*, 6(9), 1006-1021.

Zhang, M., Liu, T., Sun, H., Weng, W., Zhang, Q., Liu, C., ... & Sheng, W. (2018). Pim1 supports human colorectal cancer growth during glucose deprivation by enhancing the Warburg effect. *Cancer science*, 109(5), 1468-1479.